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FOREWORD

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INTRODUCTION

The development of human tumors is the result of multiple events that undermine the signals involved in normal growth control. The *p53* tumor suppressor gene is often mutated in the development of human cancers. An important negative regulator of *p53* function is the product of the *mdm2* oncogene. MDM2 binds and inactivates *p53* function. Amplification of *mdm2* occurs in many sarcomas and breast carcinomas suggesting an alternative mechanism of inactivating *p53*. This proposal was designed to examine the *in vivo* function of the *mdm2* oncogene in concert with the tumor suppressor *p53*. In addition, the interaction of MDM2 with other proteins and their effect on MDM2 function and tumor development were examined.

The *mdm2* gene was originally cloned from a transformed murine cell line (3T3DM) as one of three amplified genes stably maintained in the form of double minutes (1). The overexpression of *mdm2* alone in primary rat embryo fibroblasts (REFs) resulted in immortalization or in transformation when transfected with the activated *ras* gene leading to the hypothesis that *mdm2* functioned as an oncogene in the process of cell transformation (2).

Subsequently, MDM2 was discovered as a protein that bound the *p53* tumor suppressor (3). Moreover, the binding of MDM2 to *p53* inactivated *p53* function as a transcriptional activator. The interaction of MDM2 with *p53* created much excitement in the field since the *p53* tumor suppressor gene is mutated in greater than 50% of human cancers (4). Cloning and localization of the human *mdm2* gene indicated that it resides on human chromosome 12q13-14, a region often altered in sarcomas (5). Analysis of both osteosarcomas and soft tissue sarcomas revealed amplification of the *mdm2* gene in approximately one third of tumors (5, 6). Additional experiments showed amplification or overexpression of *mdm2* in glioblastomas and breast carcinomas (7-9). These data thus led to the hypothesis that overexpression of MDM2 by gene amplification represents an alternate mechanism of inactivating *p53* function.

To examine the interaction of *p53* and MDM2 *in vivo*, we deleted the *mdm2* gene in mice using homologous recombination in ES cells (10). *mdm2* null mice died during embryogenesis 5.5 days of gestation. Since one of the functions of MDM2 is the negative regulation of *p53*, we hypothesized that the embryonic lethality seen in *mdm2* homozygous mutants was due to an inability to down-regulate *p53* function. *p53* null mice are viable (11, 12) and we therefore tested our hypothesis by interbreeding mice heterozygous for both *mdm2* and *p53* genes. Strikingly, we found viable mice that were homozygous null for both *mdm2* and *p53*. Rescue of the *mdm2* $-/-$ lethality in a *p53* null background suggests that a critical *in vivo* function of MDM2 is the negative regulation of *p53* activity.

However, evidence has accumulated indicating that MDM2 has separate functions in addition to its interaction and inhibition of *p53* function. In some sarcomas, both amplification of *mdm2* and mutations in *p53* were found (13). Patients with these tumors had significantly reduced survival as compared to those with only one of these alterations. In transgenic mice, the overexpression of *mdm2* leads to an aberrant cell cycle independent of *p53* (14). These data are indicative of *p53*-independent functions. Indeed, recent developments suggest that

MDM2 binds other factors implicated in growth control. MDM2 binds another tumor suppressor, the retinoblastoma (Rb) gene product (15). This interaction also disrupts Rb function as a growth suppressor and inhibitor of transcription. In addition, a complex was detected between MDM2 and E2F1, a transcription factor important for the G1/S transition (16). In this case, however, the interaction further stimulated the activity of E2F1. Thus, MDM2 appears to inhibit Rb and p53 function, but its interaction with E2F1 suggests that in addition to inactivation of tumor suppressors, MDM2 can augment cell proliferation by activating genes involved in S-phase progression.

Our hypothesis is that MDM2 is an important component of the p53 pathway, but it has additional independent functions that affect tumorigenesis.

The specific aims of this proposal are:

- 1) to determine the mechanism of death of the *mdm2*^{-/-} embryo by investigating growth arrest and apoptosis
- 2) to examine *mdm2*^{-/-} *p53*^{-/-} mice for timing and spectrum of tumor development
- 3) to assay for other *in vivo* interactions by mating *mdm2*^{+/-} mice with *p21*^{-/-} mice and with *bax*^{-/-} mice
- 4) to analyze MDM2 function using cell lines developed from the *mdm2*/*p53* double null mice.

BODY

Experimental methods, assumptions, and procedures:

Mouse Breeding and Genotyping.

Mice null for *p53* (12) and either wild-type, heterozygous, or homozygous for the *mdm2* null allele (10) were used to study the timing and spectrum of tumor development. To generate these genotypes, mice heterozygous for the *mdm2* null mutation were crossed to normal C57BL/6J females to produce two generations of mice of mixed 129/Sv:C57BL/6 background. These mice were crossed to 129/Sv *p53* null mice (obtained from Jackson Laboratory) to generate heterozygous mice for both mutant alleles. These double heterozygote mice were crossed to each other to obtain the mice used in this tumorigenic study.

To determine mouse genotypes, DNA was extracted from tail biopsies. PCR analysis was performed using the following primer sets: for *mdm2*, (I) 5'-tgtggctggagcatgggtattg -3', (II) 5'- atctgagagctcgtgcccttcg-3', and (III) 5'-ggcggaaagaaccagctggggc-3' (10), of which (I) and (II) amplify the wild type allele while I and III amplify the mutant allele; for *p53* (I) 5'- agcgtggtgtaccttatgagc-3', (II) 5'-ggatggtgtatactcagagcc -3', and (III) 5'- gctatcaggacatagcgttggc-3' (17), of which I and II amplify the wild type allele, while II and III amplify the mutant allele; and for *bax*, (I) 5'-gggttgaccagagtggcgtagg-3', (II) 5'- gagctgatcagaacctcatgg-3', and (III) 5'-acccgcttcattgctcagcgg-3' (C.M Knudson, personal communication), of which I and II amplify the wild type allele, while I and III amplify the mutant

allele. PCR for *mdm2*, *p53* and *bax* was conducted using annealing temperatures of 65°C, 60°C, and 65°C, respectively, for 35 cycles with an extension time of 3 minutes.

Mouse embryos were digested in 1X PCR buffer (Boehringer Mannheim, Indianapolis, IN), 1 mg/ml proteinase K, and 0.1% (v/v) Triton X-100 (Sigma Chemicals, St. Louis, MO) for one hour at 55°C and the proteinase K inactivated by heating at 95°C for five minutes. One microliter was used for PCR analysis. For blastocyst genotyping, the entire volume of DNA was placed into PCR.

The presence of the BLG*mdm2* transgene was determined by a dominant coat color marker that had been coinjected with the transgene and by PCR with transgene-specific primers BLG and MDM2 as described previously (14). *E2F1* heterozygous and homozygous mutant animals were identified by PCR as described previously (18), with the following exceptions. PCR was performed for both wild-type and mutant alleles together using 16 pmol each of L26 and L28 primers and 32 pmol of L31 primer per reaction. The PCR reactions (25 µl) were amplified using AmpliTaq (Perkin-Elmer) for 1 cycle (94°C 5 min), 35 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 1 min) and 1 cycle (72°C for 7 min).

Nulliparous females of the appropriate genotypes were mated and sacrificed at day 14 or 18 of gestation or at day 10 of lactation. If necessary, pups born to BLG*mdm2* females were removed to a foster mother and replaced with slightly older pups to allow for continued nursing. Most animals were given an intraperitoneal injection of 100 µg of BrdU in PBS/g body weight approximately 2 hours before sacrifice. The first abdominal (#4) mammary glands on both sides of the animal were dissected for analysis.

Tumor analysis

Mice that developed visible tumors approximately 1 cm in diameter were sacrificed and subjected to necropsy. Mice that did not develop visible tumors but became moribund were also sacrificed and subjected to necropsy. In addition to tumor samples, tissues from heart, lung, kidney, spleen, liver, and testis were recovered. The mice were carefully examined for the presence of any other abnormalities. Brain samples were taken in those few cases from moribund mice with no apparent pathology or tumor. All the tissues were fixed in 10% buffered formalin, processed for histology, and paraffin embedded. Four-micrometer-thick paraffin sections were stained with hematoxylin and eosin. The histopathologic analysis was performed without knowledge of the genotypes of the mice.

Statistical analysis

Comparison of the tumor latency for the three strains of mice was performed using Kaplan-Meier analysis. Significant differences in the type and dissemination of tumors between mice of the three genotypes were established using the chi-square test. The statistical significance of the apoptosis in blastocysts from *mdm2* +/- x *mdm2* +/- crosses was determined using the test for equality of proportions. The statistical significance of the partial rescue of the *mdm2* null phenotype in a *bax* null background was determined using the chi square test.

TUNEL

Tdt-mediated dUTP Nick End Labeling (TUNEL) was carried out using the In Situ Cell Death POD kit (Boehringer Mannheim, Indianapolis, IN). Blastocysts were flushed from the uterus using M15 medium and the zona pellucida removed by Acid Tyrode's solution (137 mM NaCl, 3 mM KCl, 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.4 mM $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, 6 mM glucose, 1×10^{-5} M polyvinylpyrrolidone, and 0.66% (v/v) HCl). They were fixed in 4% (w/v) paraformaldehyde by a 15 minute incubation in 0.3% (v/v) Triton X-100 in 0.1% (w/v) H_2O_2 /methanol. Cells were permeabilized using 0.1% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate, then treated with terminal deoxynucleotidyl transferase and fluorescein conjugated dUTP. Labeling was detected using a peroxidase conjugated anti-fluorescein antibody and 3,3'-diaminobenzidine (DAB) (Vector Labs, Burlingame, CA).

Immunohistochemistry.

Mammary gland tissues were fixed in 0.4% paraformaldehyde in PBS at 4°C overnight, washed twice in PBS, and dehydrated through a graded series of ethanols, from 70% to 100%, according to standard procedures. The tissue was then incubated in xylene for 30 min. before it was embedded in paraffin. Sections were cut at 7- μm and placed on lysine-coated slides. After rehydration, the slides were incubated in 0.01 M citrate buffer pH 7.0 in a steamer for 25 min. for antigen retrieval and then soaked in 0.3% hydrogen peroxide in methanol for 15 min. After rinsing in PBS, the slides were blocked with serum from the Vectastain kit (Vector Labs, Burlingame, CA) and then incubated with the appropriate antibody for 1 hour at 37°C in a humidified chamber. MDM2 (9312) and cyclin E (1014) antibodies were rabbit polyclonal antisera raised by our laboratory and used at a 1:250 dilution. Cyclin A (C-19; polyclonal; 1:250) and cyclin D1 (72-13G; monoclonal; 1:100) were obtained from Santa Cruz Biotechnology. All immunohistochemistry was performed with the Elite Vectastain Kit for mouse or rabbit (Vector Labs) according to the manufacturer's instructions. Staining was detected with the substrate DAB (Vector Labs). All immunostained slides were counterstained with Nuclear Fast Red (Vector Labs) before dehydrating and mounting with Permount. BrdU immunostaining was performed using the BrdU Staining kit (Zymed, San Francisco, CA) according to the manufacturer's instructions.

Results and discussion

Our work during the last three years of funding has led to the development of two mouse models. In one model, the loss of *mdm2* results in embryonic lethality due to the inability to down modulate p53 function. Using this model, we have undertaken an examination of the critical components of the p53 pathway. In another model, the overexpression of *mdm2* in mouse mammary epithelial cells led to disruption of the cell cycle and tumor development. We have used both models to further our understanding of MDM2 function in tumorigenesis.

In the first model, the early embryonic lethality seen in *mdm2* null embryos is due to an inability to inactivate p53, and is completely rescued by the absence of p53 (10, 19). Because the MDM2/p53 interaction is critical to the survival of this *in vivo* model, it is ideal for studying

the p53 pathway, specifically the contribution of downstream effectors of p53. In the previous three years of funding, we have clearly shown the deletion of the p53 downstream target p21 had no effect on embryo lethality (20).

While an important observation, the inability of loss of *p21* to rescue the *mdm2* null phenotype did not yield any information as to the mechanism of embryonic cell death. Since the process was p53 dependent, we began to examine *mdm2* null embryos for apoptosis or cell cycle arrest, the two biological outcomes of activating p53. In order to do so, we first attempted to discern whether the few remaining cells in the empty decidua were of embryonic origin. To do this, we crossed a female mouse heterozygous for *mdm2* with a Rosa 26 male mouse which carries a ubiquitously expressed β -galactosidase gene inserted into chromosome 6 (26). We generated a male mouse heterozygous for both β -galactosidase and *mdm2*, which was then crossed with female mice heterozygous for *mdm2*. These females were sacrificed at 5.5 dpc and the deciduae treated with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). Of 30 deciduae analyzed, 25% were empty and none contained blue cells, suggesting that at 5.5 dpc the empty decidua is truly devoid of embryonic tissue (data not shown). Phenotypically normal embryos stained blue as a positive control.

To examine embryos earlier in development, *mdm2* heterozygous mice were mated and embryos collected at the blastocyst stage (3.5 dpc). Of 84 blastocysts genotyped, 21 were *mdm2* null (data not shown), the expected Mendelian ratio. To determine if *mdm2* null embryos demonstrated a phenotypic abnormality at this stage, blastocysts were examined microscopically and categorized by developmental stage (morula, unhatched blastocysts, or hatched blastocyst), then genotyped. Interestingly, of the 28 blastocysts analyzed, none that were null for *mdm2* had hatched (Fig. 1). This lack of hatching suggested that the *mdm2* null phenotype might actually begin as early as the blastocyst stage.

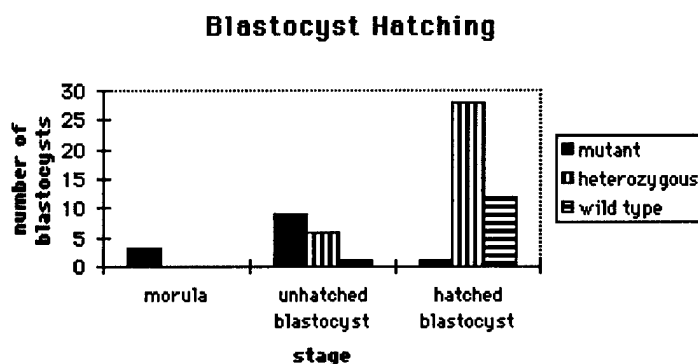


Fig. 1. Blastocysts from wild type or *mdm2* heterozygous crosses were isolated at 3.5 days post coitum, placed in culture for 24 hours and staged. After staging, they were genotyped.

Blastocysts were then tested for apoptosis using the Tdt-mediated dUTP-biotin Nick End Labeling (TUNEL) assay, which stains the nuclei of cells committed to apoptosis. Since up to ten percent of wild type blastocyst cells can undergo apoptosis (27), crosses between wild type mice were analyzed to determine if this apoptosis could be detected by the TUNEL

assay. Of the 16 wild type embryos tested, a maximum of five cells were TUNEL positive of 60-70 cells in any one blastocyst, indicating that the normal apoptotic number was less than ten percent. A blastocyst with seven or more cells undergoing apoptosis was classified as abnormal in this experiment. Of 73 blastocysts analyzed from heterozygous crosses, 17 (23.3%) demonstrated excess apoptosis. Of the 24 blastocysts from control crosses, all had <5 TUNEL positive cells per blastocyst (Table I). The affected blastocysts generally displayed much greater than seven apoptotic nuclei, ranging from ten to thirty positive nuclei in any one blastocyst (Fig. 2). Several attempts were made to genotype the blastocysts after TUNEL without success. However, only the population containing *mdm2* null embryos had abnormal levels of apoptosis making it probable that it contributes to the lethality in *mdm2* null embryos.

Table 1 - TUNEL assay in blastocysts

TUNEL	<i>Mdm2</i> +/- x <i>Mdm2</i> +/+ ^a	<i>Mdm2</i> +/- x <i>Mdm2</i> +/- ^b
Abnormal (>7)	0	17
Normal (<5)	24	56
Total	24	73

^aEmbryos from control and ^bheterozygous crosses were analyzed at 3.5 dpc by the TUNEL assay. The frequency of apoptosis in the population of embryos containing *mdm2* -/- embryos is significantly greater than the frequency in the normal population ($p < 0.01$).

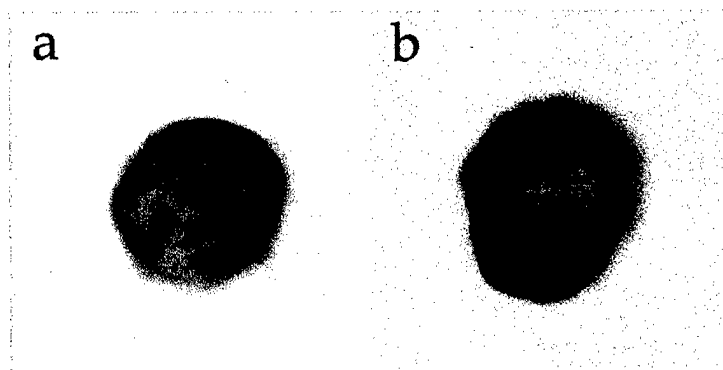


Fig 2. Blastocysts stained by TUNEL for apoptosis. a, normal embryo; b, *mdm2* null.

p53 initiates growth arrest or apoptosis through activation of its downstream target genes. One of these targets, *bax*, is a positive regulator of apoptosis. To determine if the *mdm2* null phenotype could be rescued in a *bax* null background, mice heterozygous for both *mdm2* and *bax* were generated. These mice were then mated and their offspring genotyped. From these crosses, 1 of 16 mice is expected to be null for both *bax* and *mdm2*. Of 90 mice born, none were null for both genes. Crosses between *bax* +/- *mdm2* +/- males and *bax* -/- *mdm2* +/- females were also analyzed. (Male mice null for *bax* are sterile.) From these matings, 1 of 8 pups is expected to be null for both *bax* and *mdm2*. Of 67 mice analyzed,

none were null for both genes. These data suggest that the absence of *bax* does not rescue the *mdm2* null lethality as does the absence of *p53*.

Although the absence of *bax* could not fully rescue *mdm2* null lethality, the possibility remained that the absence of *bax* could partially rescue the phenotype. To explore this possibility, *bax* +/- *mdm2* +/- males were crossed with *bax* -/- *mdm2* +/- females, and the embryos were analyzed at various stages of development. Embryos at 5.5 and 6.5 dpc were too small to be accurately genotyped, and must be examined histologically. Of 26 deciduae examined at 5.5 dpc, six or seven (25%) would be expected to be empty due to the *mdm2* -/- phenotype. However, only three deciduae, half the expected number, were found to be empty in this experiment. Of 79 embryos examined at 6.5 dpc, 67 (85%) were normal and 12 (15%) were abnormal. Twenty (25%) abnormal deciduae would be expected from this cross. The lower frequency of the *mdm2* -/- phenotype indicates that the absence of *bax* partially rescues the *mdm2* null embryo to 6.5 dpc. At 7.5 dpc, of 55 deciduae present, 45 contained embryos and 10 were empty (Table II). Genotypes were determined for 42 of the embryos, none of which was null for *bax* and *mdm2* (data not shown). These data indicate that lethality in embryos null for both *bax* and *mdm2* occurs between 6.5 and 7.5 dpc, instead of between 3.5 and 5.5 dpc as seen in embryos null for *mdm2* alone.

Table 2 - Partial rescue of the *mdm2* null phenotype in a *bax* null background

	<i>bax</i> -/- <i>mdm2</i> +/- X <i>bax</i> +/- <i>mdm2</i> +/-					
	5.5 dpc		6.5 dpc		7.5 dpc	
	Expected	Observed	Expected	Observed	Expected	Observed
Normal	23	27	59	67	41	45
Abnormal	7	3 ^a	20	12 ^a	14	10 ^b
Total		30		79		55

^aAt 5.5 and 6.5 dpc, the number of observed abnormal embryos is significantly less than expected ($p < 0.01$). ^bnot statistically significant from the expected value

To determine if these embryos were still dying by apoptosis, the TUNEL assay was performed on sections of 6.5 dpc embryos from the above cross. Of 19 embryos examined, none revealed TUNEL positive cells. As a positive control, mouse intestine which was fixed and sectioned by the same protocol stained positively in the crypt cells (data not shown.) If apoptosis were still the cause of lethality in the *mdm2* null embryos at 6.5 dpc, five embryos should have been abnormal by the TUNEL assay. This indicates that the mechanism of *mdm2* null lethality has changed in the absence of *bax*. To assess if *mdm2* null embryos are dying by growth arrest in a *bax* null background, embryo sections were stained for proliferating cell nuclear antigen, PCNA, which is a marker for DNA synthesis. Normal embryos at 6.5 dpc are proliferating and stain strongly for PCNA (Fig.3). Of the 28 embryos tested from a *bax* +/- *mdm2* +/- X *bax* -/- *mdm2* +/- cross, 22 stained strongly for PCNA. Five embryos stained only weakly and one did not stain at all (Fig. 3). The number of weakly staining embryos roughly corresponds to the number of embryos expected to be null for both

bax and *mdm2*. This weak staining may be due to the long half life of PCNA, which is 20 hours (28). Maternal tissue close to the embryos showed a variable staining pattern, ranging from very strong to very weak or negative. However, the maternal cells in the proliferating zone of the deciduae of all embryos consistently exhibited strong staining, providing an internal control for the procedure. This suggests that growth arrest and not apoptosis is the cause of *mdm2* lethality in a *bax* null background.

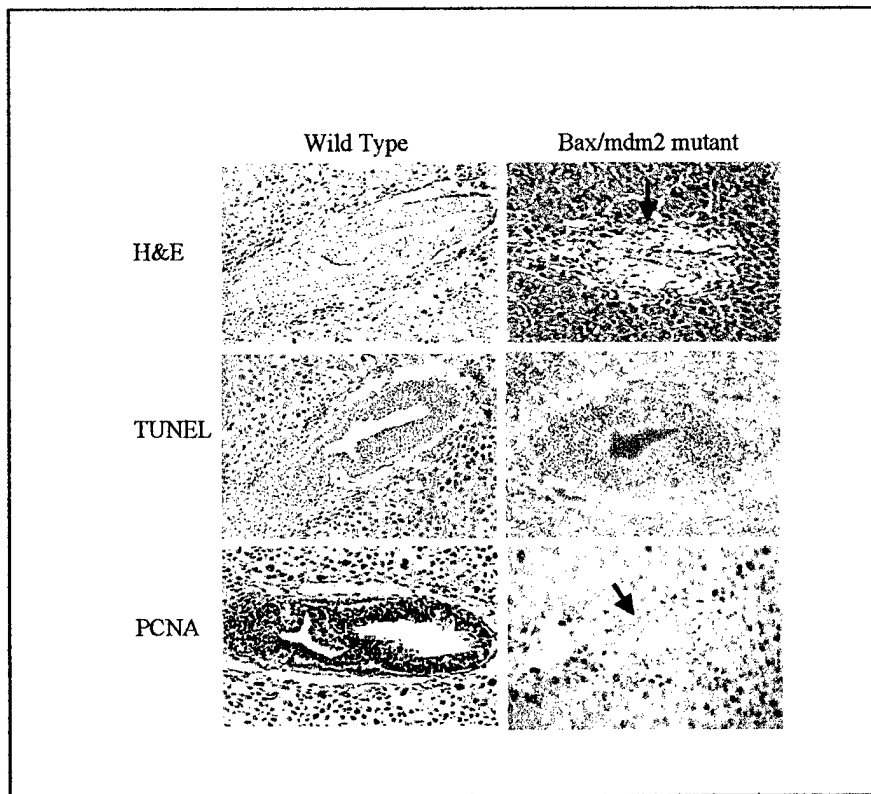


Fig. 3. Sagittal sections of 6.5 dpc deciduae from wild type crosses or from crosses between *mdm2* +/- *bax* +/- males and *mdm2* +/- *bax* -/- females. The left three panels are normal embryos stained by hematoxylin and eosin (H&E), TUNEL, or with antibodies to PCNA (top to bottom respectively). The right three panels are abnormal embryos from a cross between *mdm2* +/- *bax* +/- males and *mdm2* +/- *bax* -/- females and represent abnormal embryos probably null for both *mdm2* and *bax*. Arrows indicate the location or predicted location of the embryo.

Aim 2 of our proposal was to examine the *mdm2* -/- *p53* -/- mice for tumor development. We monitored tumour latency and spectrum in *p53* null mice in the presence or absence of *mdm2*. Two unusual findings resulted: Tumour latency in *p53* null/*mdm2* heterozygous mice was longer than in *p53*/*mdm2* double-null mice. The incidence of sarcomas was higher in *p53* null/*mdm2* heterozygous mice than in *p53* null or *p53*/*mdm2* double-null mice. These data raise the possibility that heterozygosity at the *mdm2* locus in the absence of *p53* affects the development of tumours of mesenchymal origin.

This study was described in detail in last years' report and is not described here. It has been published (29) and a reprint is attached.

The second model we are studying is one in which an *mdm2* minigene was expressed during gestation and lactation in the mammary gland of both wild-type *p53* (*p53*+/+) and *p53* knockout (*p53* -/-) mice (14). The deregulated expression of MDM2 inhibited normal

development and morphogenesis of the mammary gland, and caused hypertrophy and nuclear abnormalities. These abnormalities included both multinucleated cells and enlarged cells with giant nuclei. Analysis of DNA content showed that 30-45% of the cells were polyploid, with DNA contents up to 16N, indicating that overexpression of *mdm2* caused mammary epithelial cells to undergo multiple rounds of S-phase without cell division. This phenotype was similar in the *p53*^{+/+} and *p53*^{-/-} background, demonstrating a role for MDM2 in the regulation of DNA synthesis that is independent of the ability of MDM2 to inhibit p53 activity. Additionally, multiple lines of BLGMDM2 transgenic mice developed mammary tumors, confirming that overproduction of MDM2 contributes to tumorigenesis in epithelial cells *in vivo*. This study was published during the first year of funding (14).

The mechanism by which MDM2 overproduction disrupts the coordination of DNA synthesis in S phase and cytokinesis is unknown. However, one possible model involves the ability of MDM2 to bind and stimulate the activity of the S-phase transcription factor E2F1/DP1 (16). During the G1 phase of the cell cycle, the tumor suppressor Rb binds and inhibits E2F1 activity (30-32). E2F1 is released as a function of Rb phosphorylation by cyclin-dependent kinases late in G1, and becomes transcriptionally active. E2F1/DP1 then activates the expression of a number of genes involved in S phase, such as *cyclin E* (33), *dihydrofolate reductase* (34,35), *thymidine kinase* (36), and *DNA Pol α* (33, 37). E2F1 is a potent facilitator of DNA synthesis, as quiescent cells in tissue culture can be driven into S phase by overexpression of E2F1 alone (38-40).

As MDM2 binds and activates the S-phase-specific transcription factor E2F1, we hypothesized that increased E2F1 activity causes the development of the BLG*mdm2* phenotype. We therefore generated BLG*mdm2* mice that were null for *E2F1*. We observed no notable differences in histology or cyclin gene expression between BLG*mdm2* and BLG*mdm2*/*E2F1*^{-/-} mice, indicating that endogenous E2F1 activity was not required for the BLG*mdm2* phenotype. Since, depending on the experimental system, either loss of E2F1 function or overexpression of E2F1 results in transformation, we also tested whether overexpression of E2F1 augmented the severity of the BLG*mdm2* phenotype by generating mice bitransgenic for BLG*mdm2* and BLGE2F1. We observed a unique mixture of the two single transgenic phenotypes histologically, and found no significant changes in cyclin levels, indicating that overexpression of E2F1 had no effect on the BLG*mdm2* transgenic phenotype. Thus, increased expression or absence of E2F1 does not affect the ability of MDM2 to disrupt the cell cycle. These data were presented in detail in last years' report and have been published (41). A reprint is attached.

In order to investigate the *mdm2* null phenotype in more detail, we attempted to establish *mdm2* null embryonic stem cells in culture. However, these cells were not viable (42). Therefore, to examine the role of MDM2 in p53-mediated growth arrest or apoptosis, we took advantage of the viability of *p53*^{-/-}*mdm2*^{-/-} mouse embryo fibroblasts (MEFs) in culture (aim 4). We have established an experimentally amenable system by introducing the temperature sensitive p53 (tsp53) point mutant (p53 A135V) into *p53*^{-/-} and *p53*^{-/-}*mdm2*^{-/-} MEFs using a retroviral infection procedure. Early passage MEFs established from *p53*^{-/-} and *p53*^{-/-}*mdm2*^{-/-} embryos were infected with a retroviral vector containing the tsp53 gene and a

puromycin cassette. In order to characterize homogeneous populations of cells we isolated individual clones from the infected pools of *p53*^{-/-} and *p53*^{-/-mdm2}^{-/-} MEFs under selective conditions (2.5 µg/ml puromycin) by plating an average of 20-30 cells per 10 cm culture dish and harvesting and expanding these individual colonies. We isolated an average of 50 clones per genotype. The series of *p53*^{-/-} clones and *p53*^{-/-mdm2}^{-/-} clones were denoted K and D, respectively.

In order to quantitate p53 protein present in the different clonal populations we used the specific flow cytometric assay developed by Kastan et al., 1991 (43, 44). Each of our selected populations analyzed by FACS is of clonal origin and were gated to the respective parental lines 35-8 (*p53*^{-/-}) and 174-2 (*p53*^{-/-mdm2}^{-/-}), which are both null for *p53*. This technique allowed us to evaluate the quantitative amounts of p53 present in each line. NIH3T3 cells have endogenous wt p53 albeit at very low levels, which are not readily detectable by experimental analysis unless up regulated by irradiation or drug treatment (44). Untreated NIH3T3 cells showed, as expected, no significant levels of p53 protein by FACS (6.9%), whereas cells treated with 15J/m² of UV irradiation and harvested 24 hours post-irradiation showed an upregulation of p53 protein visible by FACS analysis (19.6%) (Fig. 4). A1-5 cells, highly overexpressing the *tsp53* protein, also show increased levels of p53 (74.2%). The clonal K and D populations examined, revealed different levels of p53 protein present within the populations (Fig. 4). For both genetic backgrounds, p53 protein levels as measured by FITC (fluorescing isothiocyanate) intensity varied from non-existent (e.g. D10 and K100) to as high as 50% (e.g. D9) when compared to the parental lines 35-8 and 174-2.

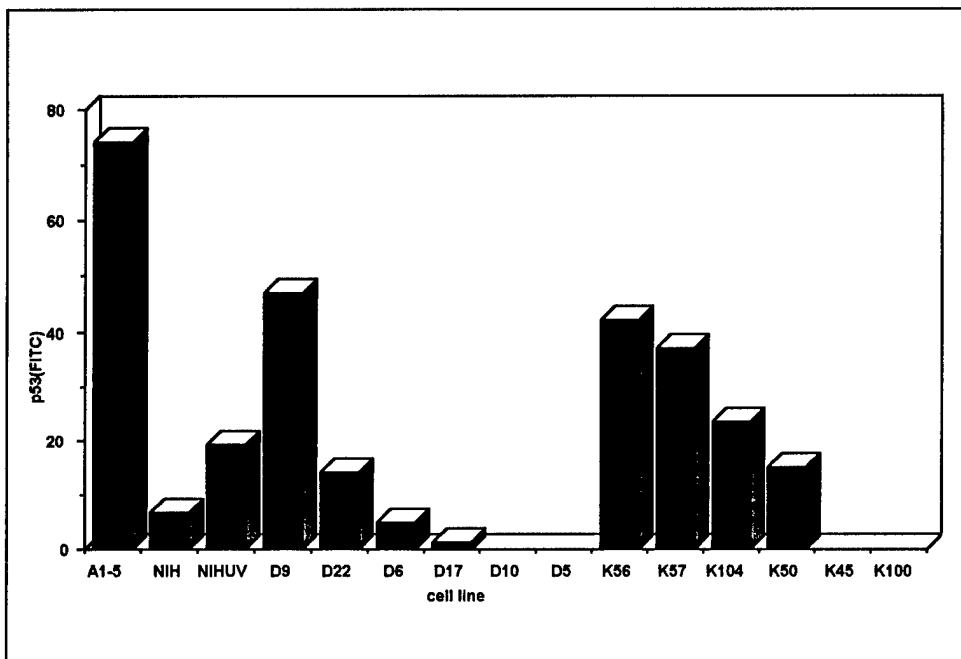


Fig. 4 Histogram showing the relative levels of p53 detected by FACS analysis using mono-clonal antibodies pAb421 and pAb248. Background levels as determined in the parental lines 35-8 (5.2%) and 174-2 (8.2%) were subtracted from the values obtained for each clonal population, respectively. As controls, A1-5 cells overexpress *tsp53* and NIH 3T3 cells have low levels of endogenous p53 (NIH), which are upregulated upon UV irradiation (NIH UV).

To verify the presence of p53 protein, we examined protein levels by immunoprecipitation. Cells were grown at 39°C (24 hours) and UV irradiated with 10J/m² since 15J/m² caused massive cell death in these cells (data not shown). At 24 hours post-irradiation, labeled cells were immunoprecipitated with a mixture of antibodies and subjected

to SDS-PAGE analysis (Fig. 5). We used wt rat embryo fibroblasts (REFs) as positive controls. Upon UV irradiation wt REFs up regulate endogenous p53 levels, which allows easy detection by experimental analysis. A 53 kDa band corresponding to p53 was detected in clones positive for p53 by FACS analysis.

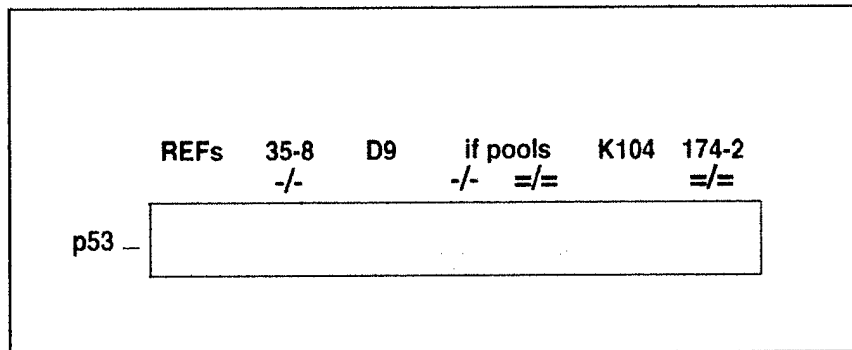


Fig. 5. Cells grown in DMEM to subconfluency were UV irradiated, labeled and immuno-precipitated with p53 monoclonal antibodies. UV-irradiated wildtype REFs were used as a positive control. Parental lines 35-8 ($p53^{-/-}$) (denoted $-/-$) and 174-2 ($p53^{-/-}mdm2^{-/-}$) (denoted $=/=$) were negative controls. The infected pools (if) are labeled $-/-$ and $=/=$.

To examine the effects of wt p53 on the growth properties of the different clones, we compared the growth characteristics of the parental lines 35-8 ($p53^{-/-}$) and 174-2 ($p53^{-/-}mdm2^{-/-}$) to selected clones at 39° C and 32° C. At 32° C, we expected to see a phenotype caused by functional wt p53, which should not occur at 39° C when p53 is mutant and nonfunctional. An equal number of cells was plated at the two temperatures and viable cells were counted at two and six days (Fig. 6). After two days in culture growth differences were noticeable in the clones lacking *mdm2*. Cell lines D9 and D22, expressing *tsp53* in an *mdm2* null background, showed a significant decrease in growth at 32° C compared to the parental line 174-2 ($p53^{-/-}mdm2^{-/-}$) (Fig. 6). This was not evident in most of the K-lines examined, although some did exhibit slower growth at 32° C (e.g. K104).

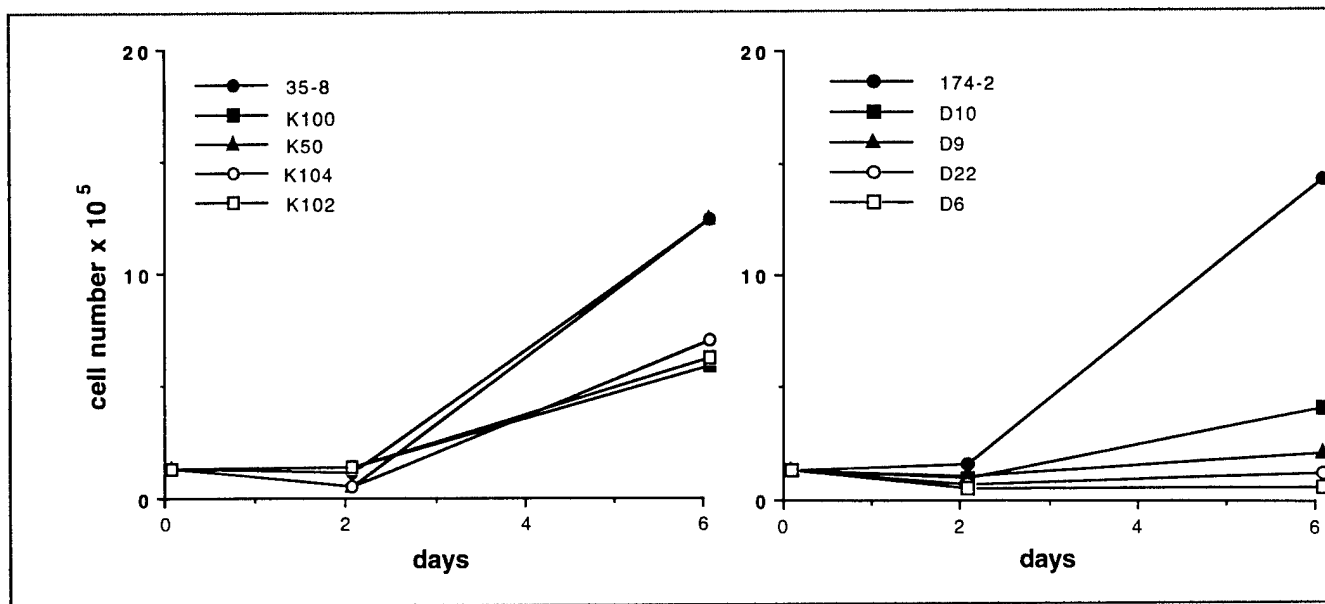


Fig. 6. Cells were grown at 32°C and counted at 2 or 6 days. All time points were performed in duplicate and the average was recorded. Parental cell lines are 35-8 ($p53^{-/-}$) and 174-2 ($p53^{-/-}mdm2^{-/-}$).

The observation that some clones containing detectable levels of p53 exhibit different growth characteristics at 32°C and 39°C led us to conduct a more detailed analysis of cell cycling behavior. Cell lines were grown at the permissive (32°C) and restrictive (39°C) temperatures and analyzed by FACS analysis. The position in the cell cycle was not significantly different between the clones expressing or lacking *mdm2*. They did not exhibit increased G1 or G2 arrest when compared to the parental lines (data not shown). However, at six days post-temperature switch, some of the double null clones (D9, D22, D6) had undergone significant apoptosis when grown at 32°C compared to the parental line 174-2 (*p53*^{-/-}*mdm2*^{-/-}) (Fig. 7). The cell line D9 exhibited 80% apoptosis when switched to 32°C.

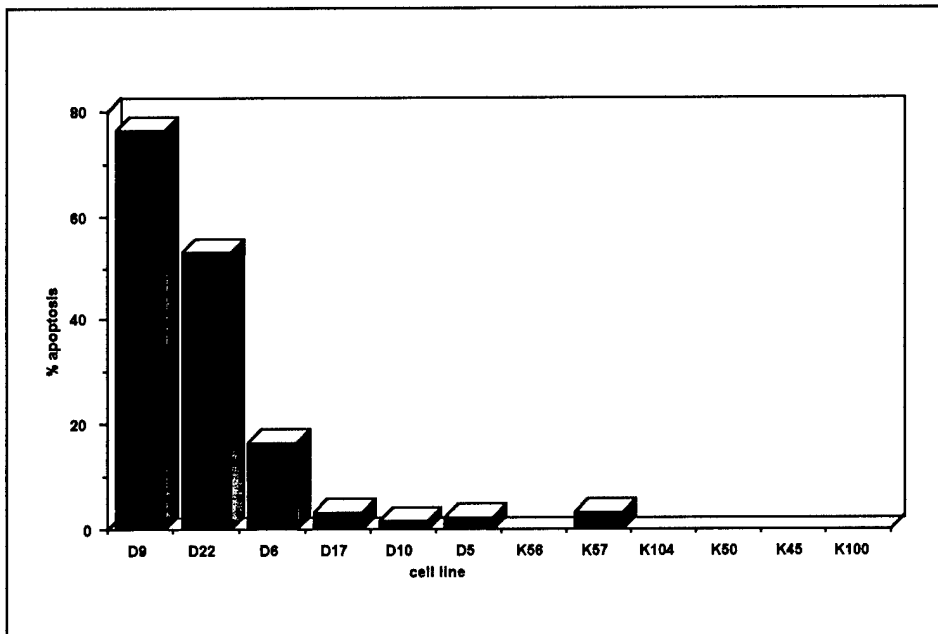


Fig. 7: Apoptosis is detected by FACS in clones lacking *mdm2*. Cells grown at 32°C for two days were harvested and analyzed by flow cytometry. Histograms show the percentage of cells in the sub-G1 fraction.

Two other cell lines lacking *mdm2* exhibited 20-60% apoptosis (e.g. D22, D6). None of the *p53*^{-/-} cell lines expressing the *tsp53* allele showed significant apoptosis. Importantly, both populations of cells express comparable levels of p53. For example, clones D9 and K56 express comparable levels of p53, yet only D9 (*mdm2*^{-/-}) showed apoptosis. Clone D6 expressed lower levels of p53 than K50 or K104. Again D6 exhibits apoptosis which K50 and K104 do not. These data suggest that the levels of p53 are not determining apoptosis. It appears that the presence or absence of the *mdm2* allele is the determining factor. In addition, the extent of apoptosis observed within the D clones was dose-dependent on the levels of p53 present. The higher the p53 amounts in a double null background, the greater the percentage of cells that underwent apoptosis (compare Fig. 4 and Fig. 7). These results suggest that apoptosis at 32°C in the D9, D22 and D6 clones (*p53*^{-/-}*mdm2*^{-/-}) is dependent on the levels of p53.

We verified apoptosis by conducting a TUNEL assay. Cells were plated at 32°C and 39°C and analyzed for apoptosis at four hours. NIH3T3 cells were treated with 1 µg/ml DNaseI to induce DNA strand breaks and used as a positive control. The D9 cell line grown at 32°C shows significant endonucleolytically cleaved DNA, a hallmark of apoptosis (data not shown), which is not present when D9 cells were grown at 39°C. Apoptosis in these clones correlates

with the reduced growth rate at 32°C (Fig. 6) as well as the presence of protein (Figs. 4 and 5). In contrast, the K104 cell line containing *mdm2* does not exhibit apoptosis at either temperature nor do the parental control lines 35-8 and 174-2.

Thus, we established a cell culture system to recreate the *in vivo* system whereby loss of *mdm2* leads to apoptosis. In order to examine the mechanism of cell death we introduced a *tsp53* allele into *p53* null and *p53/mdm2* double null MEFs from the same background. Use of the retrovirus insures single or low copy number integration and more importantly low levels of *p53* expression. In this experimental system, we compared the results of expression of *p53* in the presence or absence of *mdm2* and demonstrated that the absence of *mdm2* in MEFs leads to *p53*-dependent apoptosis. None of the clones null for only *p53*, harboring the *tsp53* gene, nor the parental lines 35-8 and 174-2 exhibited an apoptotic response when grown under identical experimental conditions. These data provide evidence that *p53* in the absence of *mdm2* induces apoptosis and recapitulate the loss of *mdm2 in vivo*. This study has been submitted for publication.

KEY RESEARCH ACCOMPLISHMENTS

- embryo lethality due to loss of *mdm2* is rescued by concomitant loss of *p53*, and is partially rescued by loss of *bax*
- loss of another downstream target of *p53*, *p21*, does not alter the *mdm2* null phenotype
- both *mdm2* null embryos and fibroblasts in culture die by apoptosis
- MDM2 overexpression in the mammary gland disrupts the cell cycle independent of *p53* and E2F1

REPORTABLE OUTCOMES

Manuscripts published

Montes de Oca Luna, R., Wagner, D.S., and Lozano, G. Deletion of *p21* cannot substitute for *p53* loss in rescue of *mdm2* null lethality. *Nature Genetics* 16:336-337.

Reinke, V., Bortner, D.M., Amelse, L.L., Lundgren, K., Rosenberg, M.P., Finlay, C.A., and Lozano, G. Overproduction of MDM2 *in vivo* disrupts S phase independent of E2F1. *Cell Growth & Differentiation* 10:147-154.

McDonnell, T.J., Montes de Oca Luna, R., Cho, S., Amelse, L., Chavez-Reyes, A., and Lozano, G. Loss of one but not two *mdm2* null alleles alters the tumour spectrum in *p53* null mice. *Journal of Pathology* 188:322-328 (1999).

Manuscripts submitted

Amelse, L.L., Chavez-Reyes, A., Montes de Oca Luna, R., Korsmeyer, S.J., and Lozano, G. Loss of *bax* inhibits *p53* apoptotic function *in vivo*. Submitted.

DeRozières, S., Maya, R., Oren, M., and Lozano, G. The loss of *mdm2* induces *p53* mediated apoptosis. Submitted.

Development of cell lines

Mdm2/p53 double null cell lines have been developed and have been distributed throughout the world.

CONCLUSIONS

The work presented in this final report makes major contributions to our understanding of p53 and MDM2. The loss of p21 cannot substitute, even partially, for loss of p53 in this the *mdm2*^{-/-} model. We have determined that embryonic lethality in *mdm2* null mice is due to apoptosis and that loss of *bax*, an inducer of apoptosis and a target of p53, partially rescues the phenotype. In addition, *mdm2* null fibroblasts expressing p53 recapitulate the apoptotic phenotype in culture and will allow a further dissection of the pathway.

An *in vivo* transgenic model was used to study the functions of MDM2. The overexpression of *mdm2* in the breast epithelium led to multiple rounds of S-phase without cytokinesis. This phenotype was independent of p53. Crosses to determine if the S-phase transcription factor, E2F1, which binds MDM2, was important indicated that the absence or overexpression of E2F1 did not affect the abnormalities seen in the mammary gland.

REFERENCES:

1. Fakharzadeh, S.S., Trusko, S.P., and George, D.L. Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *EMBO J.* 10:1565-1569, 1991.
2. Finlay, C.A. The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth. *Mol. Cell Biol.* 13:301-306, 1993.
3. Momand, J., Zambetti, G.P., Olson, D.C., George, D., Levine, A.J. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 69:1237-1245, 1992.
4. Greenblatt, M.S., Bennett, W.P., Hollstein, M., Harris, C.C. Mutations in the p53 tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis. *Cancer Res.* 54:4855-4878, 1994.
5. Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D.L., and Vogelstein, B. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature* 358:80-83, 1992.
6. Ladanyi, M., Cha, C., Lewis, R., Jhanwar, S.C., Huvos, A.G., and Healey, J.H. MDM2 gene amplification in metastatic osteosarcoma. *Cancer Res.* 53:16-18, 1993.
7. Reifenberger, G., Liu, L., Ichimura, K., Schmidt, E.E., and Collins, V.P. Amplification and overexpression of the MDM2 gene in a subset of human malignant gliomas without p53 mutations. *Cancer Res.* 53:2736-2739, 1993.
8. Sheikh, M.S., Shao, Z.M., Hussain, A., Fontana, J.A. The p53-binding protein MDM2 gene is differentially expressed in human breast carcinoma. *Cancer Res.* 53:3226-3228, 1993.
9. Quesnel, B., Preudhomme, C., Fournier, J., Fenaux, P., and Peyrat, J.-P. MDM2 gene amplification in human breast. *European J. Cancer* 30A:984-987, 1994.
10. Montes de Oca Luna, R., Wagner, D.S., & Lozano, G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* 378:203-206, 1995.
11. Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356:215-221, 1992.
12. Jacks, T., Remington, L., Williams, B.O., Schmitt, E.M., Halachmi, S., Bronson, R.T., Weinberg, R.A. Tumor spectrum analysis in p53-mutant mice. *Current Biol.* 4:1-7, 1994.

13. Cordon-Cardo, C., Latres, E., Drobnjak, M., Oliva, M.R., Pollack, D., Woodruff, J.M., Marechal, V., Chen, J., Brennan, M.F., and Levine, A.J. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. *Cancer Res.* 54:794-799, 1994.
14. Lundgren, K., Montes de Oca Luna, R., McNeill, Y. B., Emerick, E. P., Spencer, B., Barfield, C. R., Lozano, G., Rosenberg, M. P. and Finlay, C. A. 1997. Targeted expression of MDM2 uncouples S phase from mitosis and inhibits mammary gland development independent of p53. *Genes & Development* 11:714-725.
15. Xiao, Z.X., Chen, J., Levine, A.J., Modjtahedi, N., Xing, J., Sellers, W.R., Livingston, D.M. Interaction between the retinoblastoma protein and the oncoprotein MDM2. *Nature* 375:694-698, 1995.
16. Martin, K., Trouche, D., Hagemeyer, C., Sorensen, T.S., La Thangue, N.B., Kouzarides, T. Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature* 375:691-694, 1995.
17. Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T. and Tlsty, T.D. Altered cell cycle arrest and gene amplification potential accompany loss of wild type p53. *Cell* 70: 923-935, 1992.
18. Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E., and Dyson, N. J. Tumor induction and tissue atrophy in mice lacking E2F1. *Cell* 85:537-548, 1996.
19. Jones, S.N., Roe, A.E., Donehower, L.A. & Bradley, A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* 378:206-208, 1995.
20. Montes de Oca Luna, R., Amelse, L. A., Chavez-Reyes, A., Evans, S. C., Brugarolas, J., Jacks, T., and Lozano, G. Deletion of p21 cannot substitute for p53 loss in rescue of mdm2 null lethality. *Nature Genetics* 16:336-337, 1997.
21. Shiohara, M. *et al.* Absence of WAF1 mutations in a variety of human malignancies. *Blood* 84: 3781-3784, 1994.
22. Li, Y-J., Laurent-Puig, Salmon, R.J., Thomas, G., & Hameli R. Polymorphisms and probable lack of mutation in the WAF1-CIP1 gene in colorectal cancer. *Oncogene* 10:599-601, 1995.
23. Gao, X. *et al.* Somatic mutations of the WAF1/CIP1 gene in primary prostate cancer. *Oncogene* 11:1395-1398, 1995.
24. Brugarolas, J. *et al.* Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* 377:552-557, 1995.

25. Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., & Leder, P. Mice lacking p21/cip1/waf1 undergo normal development, but are defective in G1 checkpoint control. *Cell* 82:675-684, 1996.
26. Friedrich, G. and Soriano, P. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* 5:1513-1523, 1991.
27. El-Shershaby, A.M. and Hinchliffe, J.R. Cell redundancy in the zona-intact preimplantation mouse blastocyst: a light and electron microscope study of dead cells and their fate. *Embryol. Exp. Morph.* 31:643-654, 1974.
28. Bravo, R. and Macdonald-Bravo, H. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J. Cell. Biol.* 105:1549-1554, 1987.
29. McDonnell, T.J., Montes de Oca Luna, R., Cho, S., Amelse, L., Chavez-Reyes, A., and Lozano, G. Loss of one but not two *mdm2* null alleles alters the tumour spectrum in *p53* null mice. *Journal of Pathology* 188:322-328, 1999.
30. Bagchi, S., Weinmann, R., and Raychaudhuri, P. The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. *Cell* 65:1063-1072, 1991.
31. Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. The E2F transcription factor is a cellular target for the RB protein. *Cell* 65:1053-1061, 1991.
32. Chittenden, T., Livingston, D. M., and Kaelin, W. G., Jr. The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. *Cell* 65:1073-1082, 1991.
33. DeGregori, J., Kowalik, T., and Nevins, J. R. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Mol. Cell. Biol.* 15:4215-4224, 1995.
34. Blake, M. C., and Azizkhan, J. C. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. *Mol. Cell. Biol.* 9:4994-5002, 1989.
35. Mudryj, M., Hiebert, S. W., and Nevins, J. R. A role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal transduction pathway. *EMBO J.* 9: 2179-2184, 1990.

36. Dou, Q. P., Markell, P. J., and Pardee, A. B. Thymidine kinase transcription is regulated at G1/S phase by a complex that contains retinoblastoma-like protein and a cdc2 kinase. *Proc. Natl. Acad. Sci. USA* 89:3256-3260, 1992.
37. Pearson, B. E., Nasheuer, H. P., and Wang, T. S. Human DNA polymerase α gene: sequences controlling expression in cycling and serum-stimulated cells. *Mol. Cell. Biol.* 11:2081-2095, 1991.
38. Johnson, D. G., Schwartz, J. K., Cress, W. D., and Nevins, J. R. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature (Lond.)* 365:349-352, 1993.
39. Qin, X.-Q., Livingston, D. M., Kaelin, W. G., Jr., and Adams, P. Deregulated transcription factor E2F1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl. Acad. Sci. USA* 91:10918-10922, 1994.
40. Kowalik, T. F., DeGregori, J., Schwarz, J. D., and Nevins, J. R. E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. *J. Virol.* 69:2491-2500, 1995.
41. Reinke, V., Bortner, D.M., Amelse, L.L., Lundgren, K., Rosenberg, M.P., Finlay, C.A., and Lozano, G. Overproduction of MDM2 in vivo disrupts S phase independent of E2F1. *Cell Growth & Differentiation* 10:147-154.
42. McMasters K. M., Montes de Oca Luna R., Pena J. and G. Lozano. 1996. mdm2 deletion does not alter growth characteristics of p53-deficient embryo fibroblasts. *Oncogene* 13:1731-1736.
43. Kastan M. B., Radin A. I., Kuerbitz S. J., Onyekwere O., Wolkow C. A., Civin C. I., Stone K. D., Woo T., Ravindranath Y. and R. W. Craig. Levels of p53 protein increase with maturation in human hematopoietic cells. *Cancer Res.* 51:4279-4286, 1991.
44. Kastan M. B., Onyekwere O., Sidransky D., Vogelstein B. and R. W. Craig. Participation of p53 in the cellular response to DNA damage. *Cancer Res.* 51:6304-6311, 1991.

BIBLIOGRAPHY

See Reportable Outcomes section (page 17) for a complete list of manuscripts published and submitted.

MEETING ABSTRACTS

U.S. Army Medical Research and Materiel Command Breast Cancer Research Program: An Era of Hope. The regulation of p53 function by mdm2. Poster presentation by Dr. Valerie Reinke. Washington, D.C., 1997.

Texas Genetics Society. Understanding Tumorigenesis in Animal Models presentation by Dr. G. Lozano. Austin, TX, 1998.

American Association for Cancer Research, 90th Annual meeting. Meet-the-expert Sunrise Session, Mdm2 function, presentation by Dr. G. Lozano. Philadelphia, 1999.

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Deletion of *p21* cannot substitute for *p53* loss in rescue of *mdm2* null lethality

The MDM2 protein negatively regulates the activity of the p53 tumour suppressor by binding its transcriptional activation domain^{1,2}. In fact, the early embryonic lethality seen in *mdm2* null embryos is due to an inability to inactivate p53, and is completely rescued by the absence of p53 (refs 3,4). Because the MDM2/p53 interaction is critical to the survival in this *in vivo* model, it is ideal for studying the p53 pathway, specifically the contribution of downstream effectors of p53. One of the genes activated by p53 is *p21* (ref. 5). *p21* is the only known p53 target that binds and inhibits the function of the cyclin-cyclin-dependent kinase complexes and thus halts cell-cycle progression^{6,7}.

To determine the importance of the p53 target, *p21*, in rescue of the *mdm2*^{-/-} lethality, we first asked whether these genes were expressed in early development.

Northern analysis using RNA from embryonic stem cells obtained from 3.5-day blastocysts revealed that *p53* and its effectors, *mdm2* and *p21*, were highly expressed in these cells (Fig. 1a). Thus, these components of the p53 pathway were present early in development.

To examine *p21* as an effector of p53 function *in vivo*, we asked whether deletion of *p21* could substitute for deletion of *p53* in the rescue of *mdm2*^{-/-} embryos. Mice heterozygous for *mdm2* and null for *p21* were mated with each other^{3,8}. In this cross, 25% of the mice are expected to be double null. Of 46 mice born, 11 (24%) were *mdm2*^{+/-} *p21*^{-/-}, 35 (76%) were *mdm2*^{+/-} *p21*^{-/-} and none were *mdm2*^{-/-} *p21*^{-/-}. These data indicate that the loss of *p21*, unlike the loss of *p53*, does not rescue the lethality of *mdm2*^{-/-} embryos.

The possibility remained that loss of *p21*

was insufficient to allow *mdm2*^{-/-} embryos to survive until birth, but that a partial rescue occurred. Again, *mdm2*^{+/-} *p21*^{-/-} mice were mated with each other, and pregnant females were examined during gestation. Genotyping of embryos at days 9.5 and 7.5 of gestation did not yield any double null embryos (Fig. 1b). To address the possibility that rescue of *mdm2*^{-/-} lethality at 5.5 days of development by loss of *p21* delayed death by a very short time, the deciduae containing embryos from a cross between *p21*^{-/-} *mdm2*^{+/-} mice were dissected at 5.5 days of gestation and paraffin embedded. Of 25 deciduae sectioned, five (20%) showed lack of embryo development reminiscent of the empty deciduae seen in *mdm2* heterozygous crosses³ (Fig. 2a,b). Thus, loss of *p21*, in contrast to *p53* deletion, did not even partly rescue the lethality of *mdm2*^{-/-} embryos.

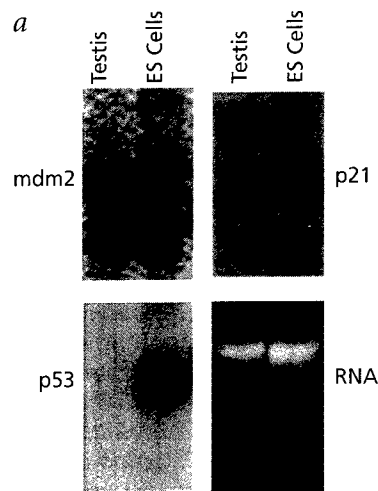


Fig. 1 a, RNA isolated from testes and ES cells was hybridized with the *mdm2*, *p53* and *p21* probes. The ethidium-stained RNA gel is shown (bottom panel) to indicate appropriate loading. **b**, Genetic analysis of *p21*/*mdm2* embryos. Only the *mdm2* genotype is shown, as all mice from this cross were *p21* null. The abnormality seen in this cross is an empty decidua. na, not applicable; nd, not determined.

<i>p21</i> ^{+/+} <i>mdm2</i> ^{+/+} × <i>p21</i> ^{-/-} <i>mdm2</i> ^{+/+}					
stage	phenotype		genotype		
	normal	abnormal	<i>mdm2</i> ^{+/+}	<i>mdm2</i> ^{+/-}	<i>mdm2</i> ^{-/-}
E9.5	13	1	7	6	0
E7.5	30	6	9	21	0
E5.5	20	5	nd	nd	nd
total	63 (84%)	12 (16%)	16 (37%)	27 (63%)	0
E3.5	na	na	18 (22%)	43 (52%)	22 (26%)

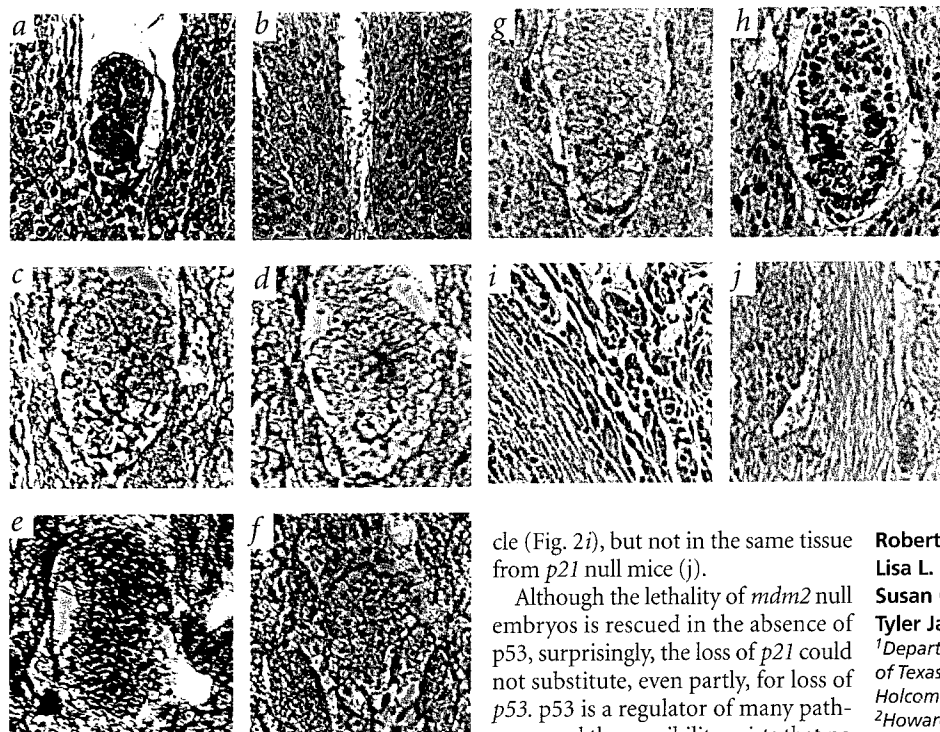


Fig. 2 The phenotype and expression of several genes were assayed in mouse embryos at 5.5 days post coitum. Deciduae from crosses between *p21*^{+/+} *mdm2*^{+/+} mice were dissected, paraffin embedded and sectioned. While most embryos were normal (**a**), 20% of the embryos had an abnormal phenotype (**b**). To assay for *p21* induction by *p53*, normal embryos were treated with 5.0 Gy of ionizing radiation and hybridized *in situ* with anti-sense (**c**) and sense (**d**) *p21* probes. As a control for induction of the *p53* pathway, *in situ* hybridization with antisense (**e**) and sense (**f**) *mdm2* probes was performed on irradiated embryos. Anti-*p21* (**g**) and anti-*mdm2* antibodies (**h**) were used to detect protein. As controls, anti-*p21* antibodies were also used on uteri of normal (**i**) and *p21* null mice (**j**).

We further examined the ability of *p53* to activate transcription in this system. We analysed induction of *p53* protein, and *p21* and *mdm2* mRNAs in embryos at 5.5 days of development after irradiation with 5.0 Gy. In embryos, *p53* was induced by 100 minutes (data not shown), with no induction of *p21* mRNA (Fig. 2c,d). In contrast, another transcriptional target of *p53*, the *mdm2* gene⁹⁻¹¹, was induced by gamma radiation in both the embryo and surrounding maternal tissue of the decidua (Fig. 2e,f, respectively). Anti-*p21* and -*mdm2* antibodies were used to detect protein. The *p21* antibody did not detect protein, whereas MDM2 was clearly produced in these embryos (Fig. 2g,h, respectively). As controls, the *p21* antibody was able to detect *p21* in uterine mus-

cle (Fig. 2i), but not in the same tissue from *p21* null mice (j).

Although the lethality of *mdm2* null embryos is rescued in the absence of *p53*, surprisingly, the loss of *p21* could not substitute, even partly, for loss of *p53*. *p53* is a regulator of many pathways, and the possibility exists that no single target can affect the functions of *p53*—a possibility supported by the lack of mutations in *p21* in human tumours¹²⁻¹⁴ and the lack of susceptibility of *p21* null mice to tumorigenesis^{8,15}. However, the lack of *p21* induction by *p53* in wild-type embryos at 5.5 days of development indicates that *p21* is not part of this pathway. Thus, we have unveiled an *in vivo* situation in which the ability of *p53* to exert its effects is not dependent on activation of *p21*.

Acknowledgements

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- Chen, J., Marechal, V. & Levine, A.J. *Mol. Cell. Biol.* **13**, 4107-4114 (1993).
- Oliner, J.D. et al. *Nature* **362**, 857-860 (1993).
- Montes de Oca Luna, R., Wagner, D.S. & Lozano, G. *Nature* **378**, 203-206 (1995).
- Jones, S.N., Roe, A.E., Donehower, L.A. & Bradley, A. *Nature* **378**, 206-208 (1995).
- El-Diery, W.S. et al. *Cell* **75**, 817-825 (1993).
- Harper, W.J., Adami, G.R., Wei, N., Keyomarsi, K. & Elledge, S.J. *Cell* **75**, 805-816 (1993).
- Xiong, Y. et al. *Nature* **366**, 701-704 (1993).
- Brugarolas, J. et al. *Nature* **377**, 552-557 (1995).
- Barak, Y., Juven, T., Haffner, R. & Oren, M. *EMBO J.* **12**, 461-468 (1993).
- Juven, T., Barak, Y., Zauberman, A., George, D.L. & Oren, M. *Oncogene* **8**, 3411-3416 (1993).
- Wu, X., Bayle, J.H., Olson, D. & Levine, A.J. *Genes Dev.* **7**, 1126-1132 (1993).
- Shiohara, M. et al. *Blood* **84**, 3781-3784 (1994).
- Li, Y.-J., Laurent-Puig, Salmon, R.J., Thomas, G. & Hameli, R. *Oncogene* **10**, 599-601 (1995).
- Gao, X. et al. *Oncogene* **11**, 1395-1398 (1995).
- Deng, C., Zhang, P., Harper, J.W., Elledge, S.J. & Leder, P. *Cell* **82**, 675-684 (1996).

Overproduction of MDM2 *in Vivo* Disrupts S Phase Independent of E2F1¹

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Abstract

Expression of a β -lactoglobulin (BLG)/*mdm2* transgene (BLG*mdm2*) in the epithelial cells of the mouse mammary gland causes an uncoupling of S phase from M phase, resulting in polyploidy and tumor formation. The cell cycle defects are independent of interactions with p53. Because MDM2 also binds and activates the S phase-specific transcription factor E2F1, we hypothesized that increased E2F1 activity causes the development of the BLG*mdm2* phenotype. We, therefore, generated BLG*mdm2* mice that were null for *E2F1*. We observed no notable differences in histology or cyclin gene expression between BLG*mdm2* and BLG*mdm2*/*E2F1*^{-/-} mice, indicating that endogenous E2F1 activity was not required for the BLG*mdm2* phenotype. Because, depending on the experimental system, either loss of E2F1 function or overexpression of E2F1 results in transformation, we also tested whether overexpression of E2F1 augmented the severity of the BLG*mdm2* phenotype by generating mice that were bitransgenic for BLG*mdm2* and BLG*E2F1*. We observed a unique mixture of the two single transgenic phenotypes histologically and found no significant changes in cyclin levels, indicating that overexpression of E2F1 had no effect on the BLG*mdm2* transgenic phenotype. Thus, increased expression or absence of E2F1 does not affect the ability of MDM2 to disrupt the cell cycle.

Introduction

The most well understood function of the protein encoded by the *mdm2* oncogene is its ability to bind and inactivate the p53 tumor suppressor (1). *mdm2* is also a transcriptional

target of p53 (2, 3), and it thereby completes a feedback loop for the regulation of p53 activity. Genetic experiments in the mouse have illustrated the functionally significant relationship of these two molecules. The early embryonic lethality of mice lacking *mdm2* is completely rescued by deletion of *p53* (4, 5). These data demonstrate that MDM2 is both necessary and sufficient to regulate p53 activity in early mouse development. However, these experiments cannot address possible additional functions of MDM2 because the *mdm2*-deficient mouse dies at 5.5 days of embryogenesis.

Other lines of evidence indicate that MDM2 does, indeed, have a function in addition to regulation of p53. Most human tumors with an amplified *mdm2* gene retain a wild-type *p53*, indicating that overexpression of MDM2 is sufficient to abrogate p53 activity (6). However, tumors that contain both a *p53* mutation and *mdm2* amplification have been identified and are associated with a worse prognosis than are tumors with a single alteration, indicating that a dual mutation can provide an additional growth advantage (7). Additionally, overexpression of MDM2 in tissue culture cells that lack p53 confers a transformation phenotype on those cells (8).

Finally, and most convincingly, overexpression of *mdm2* *in vivo* leads to disruption of the normal cell cycle, independent of p53 (9). In these experiments, the BLG⁴ promoter, which is active only in the pregnant and lactating mammary gland (10), was used to drive expression of an *mdm2* transgene (BLG*mdm2*) in the mouse. Mammary glands from lactating female transgenic mice displayed abnormal development, marked histologically by the presence of abnormally large, polyploid epithelial cells. Additionally, these epithelial cells still synthesized DNA at a time when the cells of a normal gland would have ceased to proliferate and been fully differentiated. Most remarkably, this phenotype occurred even in a *p53*^{-/-} background, indicating that increased MDM2 levels can affect cell proliferation and differentiation in this tissue independent of p53.

The mechanism by which MDM2 overproduction disrupts the coordination of DNA synthesis in S phase and cytokinesis is unknown. However, one possible model involves the ability of MDM2 to bind and stimulate the activity of the S-phase transcription factor E2F1/DP1 (Fig. 1; Ref. 11). During the G₁ phase of the cell cycle, the tumor suppressor Rb binds and inhibits E2F1 activity (12–14). E2F1 is released as a function of Rb phosphorylation by cyclin-dependent kinases late in G₁ and becomes transcriptionally active (15, 16). E2F1/DP1 then activates the expression of a number of genes involved in S phase, such as *cyclin E* (17), *dihydrofolate reductase* (18, 19), *thymidine kinase* (20), and *DNA Pol*

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⁴ The abbreviations listed are: BLG, β -lactoglobulin; BrdUrd, bromodeoxyuridine.

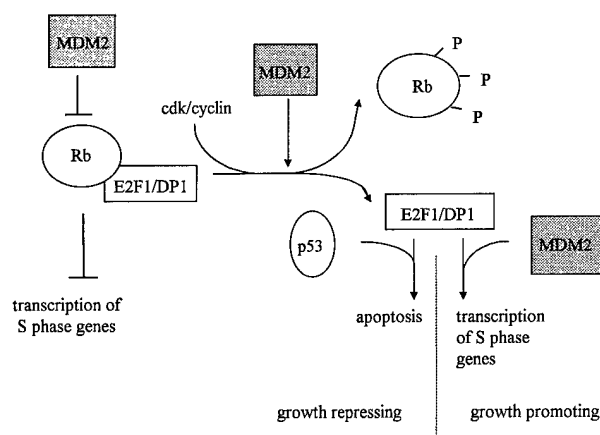


Fig. 1. Model for MDM2 involvement in the Rb/E2F1 pathway. MDM2 might prevent E2F1 function during G₁-phase transcriptional repression when associated with Rb, influence the Rb/E2F1 interaction itself, or promote the ability of free E2F1 to activate S-phase gene transcription.

α (17, 21). E2F1 is a potent facilitator of DNA synthesis, as demonstrated by the driving of quiescent cells into S phase by overexpression of E2F1 alone (22–24).

The creation of mice deficient for *E2F1* provides support for an opposing function of E2F1. Mice lacking *E2F1* are predisposed to hyperplasia and tumor development in certain tissues (25, 26). These data indicate that E2F1 can actually act as a negative growth regulator *in vivo*. The molecular mechanism for this activity is unknown but has been attributed to the fact that E2F1 can promote p53-dependent apoptosis (23, 24, 27). Lack of p53-dependent apoptosis in *E2F1*-deficient mice (26) might accelerate tumor formation. Additionally, E2F1, when complexed with Rb during the G₁ phase of the cell cycle, can mediate the transcriptional repression of S-phase genes (28–31). E2F1, thus, provides both positive and negative control of S-phase entry.

Because MDM2 binds E2F1 and stimulates its activity in S phase, we asked whether this interaction was responsible for the cell cycle defects induced by MDM2 overexpression in mammary epithelial cells. To test *in vivo* the hypothesis that the BLG*mdm2* phenotype was due to increased E2F1-mediated transactivation of S-phase genes, we generated mice carrying the BLG*mdm2* transgene in an *E2F1* null background. Histological analysis and examination of S phase demonstrated that E2F1 is not required for the development of the BLG*mdm2* phenotype. In addition, we generated bi-transgenic mice by crossing mice that overexpress a BLG*E2F1* transgene in mammary epithelial cells, which causes hyperplasia and increased apoptosis, with BLG*mdm2* mice. The mammary glands in mice overexpressing both the *E2F1* and *mdm2* transgenes showed a combination of the individual phenotypes histologically and did not demonstrate a significant change in the amount of inappropriate DNA synthesis. The above experiments define an alternative pathway for MDM2 function *in vivo*, in addition to known interactions with p53 and E2F1.

Results

Increased Cyclin A but not Cyclin D or E Expression in BLG*mdm2* Mammary Tissue.

The phenotype of the BLG*mdm2* transgenic mouse was initially identified as a lack of cellular proliferation in the mammary gland during pregnancy and lactation (9). Additionally, a large percentage of the cells in the gland had enlarged, often multiple, nuclei and were polyploid. This phenotype was most apparent at mid-lactation, a time when the wild-type gland has already completed proliferation and differentiation and is at full capacity for milk production. In contrast, the BLG*mdm2* transgenic gland produced little milk, and the epithelial cells also displayed signs of continued DNA synthesis, as determined by BrdUrd incorporation (Fig. 2). To elucidate the molecular nature of the transgenic phenotype, we asked whether there were any alterations in the production of various cell cycle-regulated proteins at different stages of mammary gland development. Because cyclin expression is tightly regulated in various phases of the cell cycle, we focused on cyclin D1 (G₁), cyclin E (G₁-S), cyclin A1 (S-G₂), and cyclin B1 (G₂-M). To that end, we performed immunohistochemistry on wild-type and BLG*mdm2* transgenic mammary glands at mid-pregnancy and midlactation, using antibodies raised against the various cyclins.

Two G₁ cyclins tested, cyclin D1 and cyclin E, showed similar production patterns in both wild-type and transgenic mammary glands. Specifically, at day 14 of pregnancy, when growth factors were stimulating cyclin D1 production (15), mammary epithelial cells from both wild-type and transgenic mice were positive for cyclin D1 (Fig. 2). This expression decreased somewhat in animals of either genotype by day 10 of lactation, when the cells were no longer receiving proliferation signals from the extracellular matrix (Fig. 2). Cyclin E, a G₁-S phase cyclin, was produced in most cells of the mammary gland of either genotype at day 14 of gestation (data not shown) or at day 10 of lactation (Fig. 2).

Immunohistochemistry performed with an antibody to the S-G₂-specific cyclin A revealed an interesting difference between the wild-type and the BLG*mdm2* transgenic mammary gland. Cyclin A was detectable in both tissues during pregnancy, but during lactation, only the BLG*mdm2* transgenic gland continued to be positive for cyclin A (Fig. 2).

Two different cyclin B antibodies were used to measure the levels of cyclin B in the epithelial cells of the BLG*mdm2* transgenic mice. These antibodies only weakly stained a positive control. Therefore, we stained mammary epithelial cells of BLG*mdm2* transgenic mice with two other antibodies for mitosis specific proteins, namely, MPM-2 and histone H3 phosphorylation (32, 33). Both antibodies positively stained the mammary epithelial cells of BLG*mdm2* transgenic mice at day 10 of lactation (Fig. 3). The histone H3 phosphorylation antibody was comparable to the control sample at day 18 of gestation, whereas the MPM-2 antibody was weaker compared to day 14 of gestation, when most of the mammary epithelial cells are cycling. These data suggest that the cells of the mammary gland continue to make mitosis-specific proteins at a time point when they should be fully differentiated.

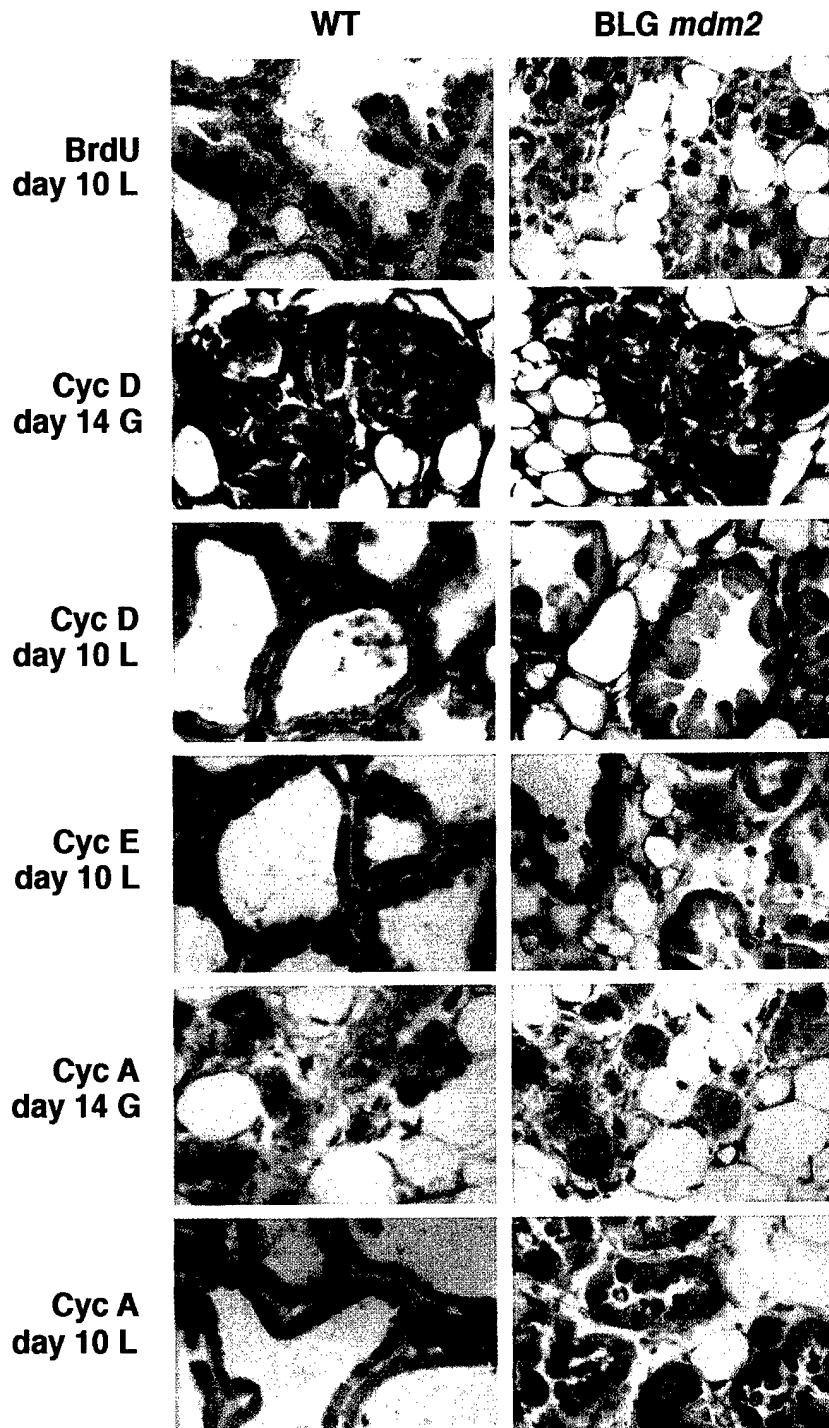


Fig. 2. Analysis of cell cycle gene expression in wild-type (WT) or BLG*mdm2* transgenic mammary glands. Wild-type and BLG*mdm2* mammary glands at day 10 of lactation were stained with H&E and photographed at $\times 200$ magnification. Immunohistochemical analysis was performed on wild-type or BLG*mdm2* mammary glands to measure the levels of BrdUrd and cyclins D, E, and A. All immunohistochemical analysis was performed on mammary glands taken at day 10 of lactation, with the addition of samples at day 14 of gestation for cyclin D and cyclin A. Microscopy for all immunohistochemistry was performed at $\times 400$ magnification.

BLG*mdm2* and BLG*mdm2*/E2F1^{-/-} Mammary Glands Are Histologically Similar. Mice carrying the BLG*mdm2* transgene demonstrate a p53-independent function for MDM2. Specifically, in the presence or absence of p53, the mammary epithelial cells of BLG*mdm2* transgenic mice are hypoproliferative and become large, multinucleate, and polyploid. Because E2F1 is bound and activated by MDM2 in

tissue culture (11), we examined whether E2F1 contributed to the transgenic phenotype (see Fig. 1 for model). BLG*mdm2* mice were crossed with mice heterozygous for a null allele of *E2F1* (25). BLG*mdm2*/E2F1^{+/-} F₁ progeny were then backcrossed to E2F1^{+/-} mice. The resulting BLG*mdm2*/E2F1^{-/-} females were mated and sacrificed at day 10 of lactation. The mammary glands of these mice were then

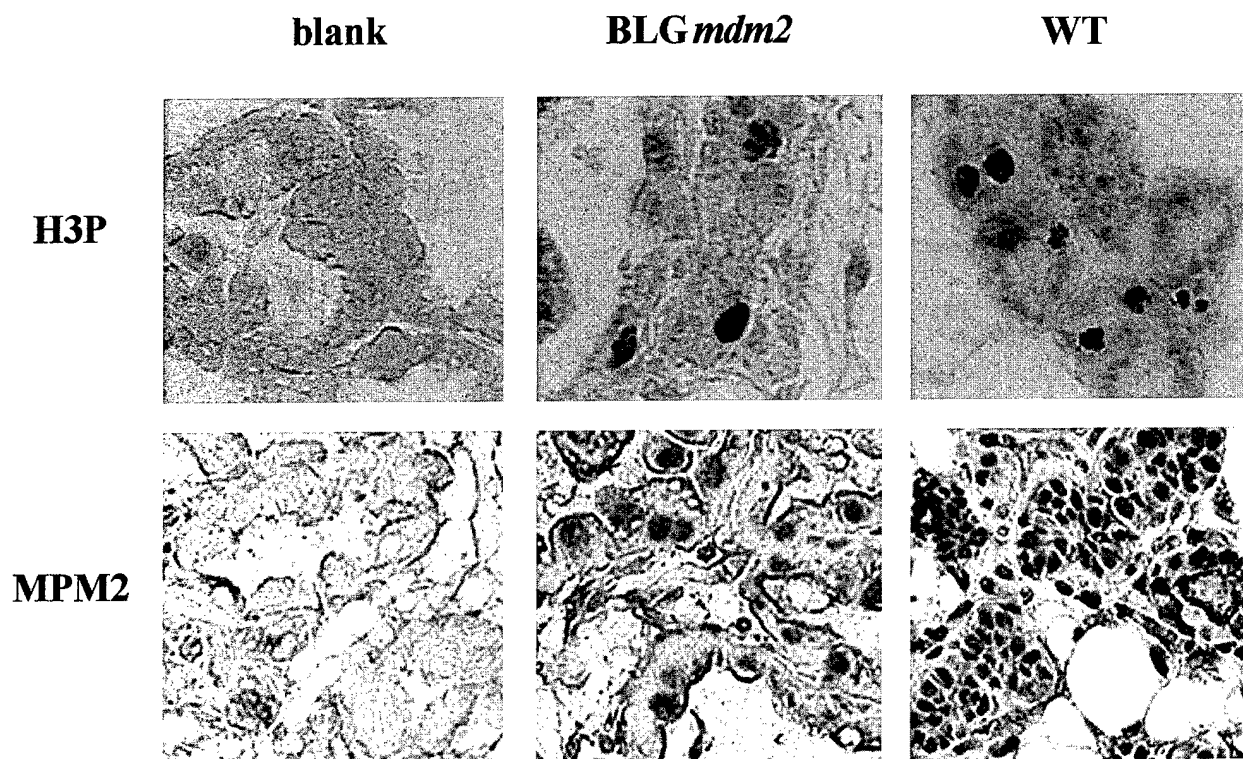


Fig. 3. Analysis of mitotic gene expression in wild-type (WT) or BLGmdm2 transgenic mammary glands. Epithelial cells of the mammary glands of the BLGmdm2 transgenic mice were stained with antibodies to histone H3 phosphorylation (H3P) or MPM-2. Negative normal controls (blank) and BLGmdm2 sections were taken at day 10 of lactation. Positive controls were from day 14 (MPM-2) or 18 (H3P) of gestation.

compared with the mammary glands of wild-type, BLGmdm2, and *E2F1*^{-/-} mice to determine whether there were histological differences, changes in cyclin gene expression, and BrdUrd incorporation, which shows the extent of DNA synthesis.

The general hypoplasia and enlarged or multiple nuclei present in BLGmdm2 transgenic mice were also present in BLGmdm2 transgenic *E2F1* null mice but not in wild-type or *E2F1* null mice (Fig. 4). Additionally, the extent of DNA synthesis was roughly equivalent in tissues carrying the BLGmdm2 transgene regardless of the *E2F1* genotype (Fig. 4 and Table 1). In contrast, the wild-type and *E2F1*^{-/-} cells of the mammary glands, which are histologically similar, showed virtually no BrdUrd incorporation (Fig. 4 and Table 1). Investigation into changes in expression of cyclin genes yielded no differences between genotypes, with the exception of cyclin A. As in the BLGmdm2 tissue, cyclin A was produced in some but not all of the BLGmdm2/*E2F1*^{-/-} cells, whereas there was no appreciable cyclin A production in the wild-type and *E2F1*^{-/-} cells (Fig. 4). Surprisingly, cyclin E levels were not decreased in the absence of *E2F1*, as might be expected of an *E2F1* transcriptional target (Fig. 4; Ref. 17). Because cyclin A overexpression has been associated with apoptosis (34), we also used terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling to measure apoptosis. BLGmdm2/*E2F1*^{-/-} mammary epithelial cells were terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling negative, as were BLGmdm2 transgenic mice

(9). These data demonstrated that there was no significant difference between BLGmdm2 transgenic glands in the presence or absence of *E2F1*.

Overexpression of E2F1 Does Not Alter the Effects of MDM2 Overproduction. To extend the above observations, we further tested the hypothesis of E2F1 involvement in the BLGmdm2 phenotype. If the ability of E2F1 to promote S phase is increased in the presence of MDM2, then simultaneous overproduction of E2F1 and MDM2 should exacerbate the BLGmdm2 phenotype. To test this possibility, we used mice that overexpress an *E2F1* transgene in the mammary gland (BLGE2F1) and display a hyperproliferation of cells and increased apoptosis.⁵

To create mice that overproduce both E2F1 and MDM2, we crossed mice carrying the BLGE2F1 transgene with BLGmdm2 transgenic mice. Females carrying one allele of each transgene were sacrificed 10 days after giving birth, and the mammary glands in these bitransgenic mice were compared with those from mice carrying either of the two single transgenes. Histological examination of H&E-stained sections indicated that the bitransgenic mammary tissue had a unique mixture of the two single transgenic phenotypes (Fig. 5). Both small, hyperproliferative cells, indicative of E2F1 overproduction, and large, multinucleate cells, indicative of MDM2 overproduction, were apparent. Additionally,

⁵ D. M. Bortner, unpublished observations.

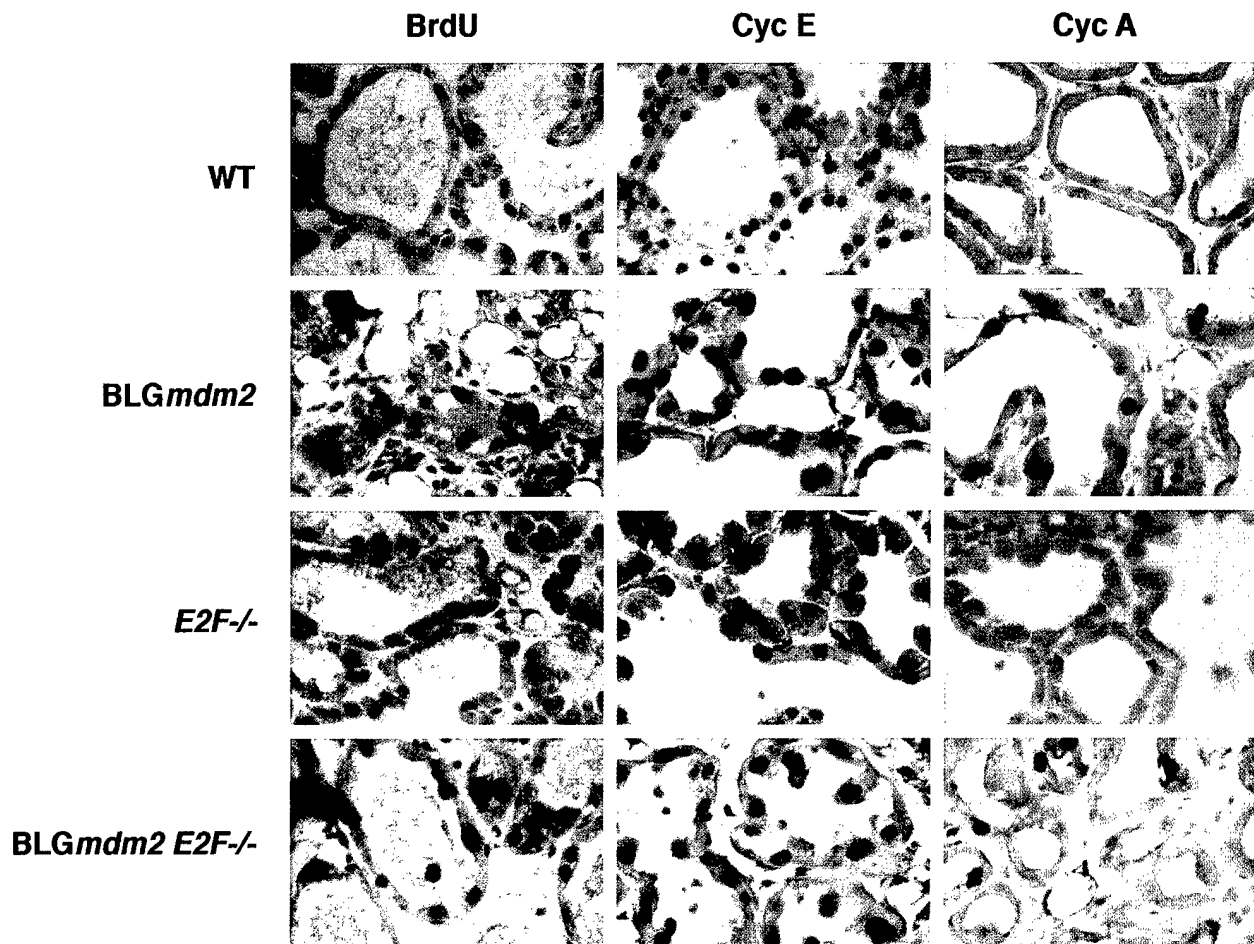


Fig. 4. Histological comparison between *BLGmdm2* and *BLGmdm2/E2F1*^{-/-} mammary glands. Mammary glands were taken at day 10 of lactation from mice with the following genotypes: wild-type (*WT*), *BLGmdm2*, *E2F1*^{-/-}, and *BLGmdm2/E2F1*^{-/-}. The glands were then fixed, embedded, and sectioned. Immunohistochemical analysis for BrdUrd, cyclin E, and cyclin A was performed on sections of glands from mice of each genotype and photographed at $\times 400$ magnification.

Table 1 Quantitation of BrdUrd incorporation^a

Genotype	No. of BrdUrd-positive cells/total no. of cells ^b	% BrdUrd-positive cells
Wild-type	3/734	0.4
<i>BLGmdm2</i>	62/677	9.2
<i>E2F1</i> ^{-/-}	1/304	0.3
<i>BLGmdm2/E2F1</i> ^{-/-}	25/239	10.4
<i>BLGE2F1</i>	43/563	7.6
<i>BLGmdm2/BLGE2F1</i>	37/516	7.2

^a Sections of mammary glands taken at day 10 of lactation from mice with the indicated genotypes were stained for BrdUrd incorporation.

^b Cells from two to five fields for each genotype were counted, and the number of BrdUrd-positive cells was divided by the total number of cells to determine the percentage of BrdUrd-positive cells.

inappropriate DNA synthesis, as measured by BrdUrd incorporation, was present in bitransgenic mammary tissue at levels similar to those of either single transgenic gland (Fig. 5 and Table 1). No changes in the levels of cyclins A or E were observed in the bitransgenic compared to the single transgenic glands (Fig. 5), although increased cyclin E might be

expected in the glands of mice carrying the *BLGE2F1* transgene. Thus, these data indicate that increased levels of *E2F1* did not alter the phenotype of *BLGmdm2* transgenic glands and further support the finding that *E2F1* function is not required for the cell cycle defects in *BLGmdm2* transgenic mammary glands.

Discussion

Transgenic mice overproducing MDM2 exhibit a disruption of normal cellular proliferation in the mammary gland, in a p53-independent manner (9). This phenotype is marked by a decrease in mammary epithelial cell number, and the cells tend to contain enlarged or multiple nuclei that undergo multiple rounds of inappropriate DNA synthesis and can become polyploid. A molecular explanation for this phenotype is difficult to formulate because very little is known about MDM2 function outside of its ability to inhibit p53 function. The first step in further characterization of the *BLGmdm2* phenotype was to identify the defective cell cycle stage by investigating cyclin gene expression, which pinpointed S phase as a likely candidate.

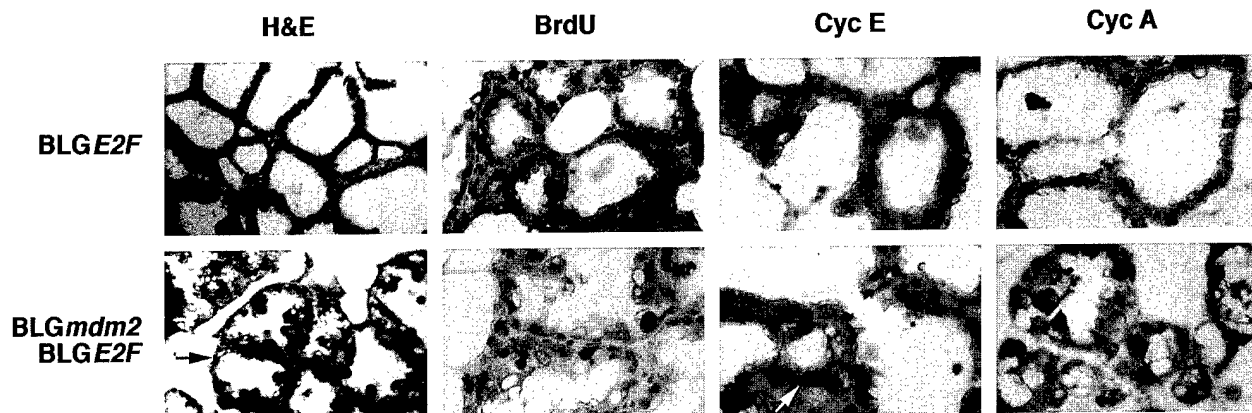


Fig. 5. Histological comparison between BLGE2F1 and BLGmdm2/BLGE2F1 mammary glands. Mammary glands taken from mice transgenic for either BLGE2F1 or BLGmdm2/BLGE2F1 at day 10 of lactation were fixed, embedded, and sectioned. H&E staining as well as immunohistochemical analysis for BrdUrd, cyclin E, and cyclin A were performed. H&E sections were photographed at $\times 200$ magnification, and all sections for immunohistochemical analysis were photographed at $\times 400$ magnification. Arrows highlight the small hyperproliferative cells indicative of E2F1 overproduction (black arrow) and the large multinucleate cells indicative of MDM2 overproduction (white arrow).

Analysis of cyclin gene expression in the mammary epithelial cells of wild-type mice in comparison to the BLGmdm2 transgenic mice only revealed significant differences in cyclin A levels. Cyclin A was produced at high levels in many cells of the transgenic mammary gland but not the wild-type gland. These data reinforce the notion that DNA synthesis is aberrant in the MDM2-overexpressing cells and indicate that the cells are either stranded in S phase, perhaps because they are unable to recognize an exit signal, or are hypersensitive to signals which cause them to enter S phase inappropriately. Interestingly, a transgenic mouse overexpressing cyclin A in the mammary gland has been generated using the same BLG promoter used in this study (34). The overexpression of cyclin A caused nuclear abnormalities such as multinucleation and karyomegaly and an increased number of apoptotic cells as compared to normal mammary epithelial cells. The absence of apoptosis in the mammary epithelial cells of the BLGmdm2 transgenic mice described here, even in the presence of high levels of cyclin A, may be due to the ability of MDM2 to inhibit p53-dependent apoptosis (35).

Rather surprisingly, cyclin E was present in virtually every epithelial cell of either wild-type or transgenic glands, possibly because the cells are arrested (or trying to arrest, in the case of the BLGmdm2 transgenic gland) at the G₁ phase of the cell cycle or because cyclin E levels do not noticeably oscillate in this tissue.

Several proteins besides p53 have been identified that interact with MDM2. MDM2 binds p19^{ARF}, one of the proteins encoded by the *INK4a* locus (36–38). Because this interaction causes the degradation of MDM2, it is unlikely to be responsible for the phenotype of the BLGmdm2 mice, which is due to the overproduction of MDM2. MDM2 has more recently been shown to bind Numb, a protein that participates in cell fate specification (39), but the functional significance of this interaction is not clear. The interaction of MDM2 with two other proteins, Rb and E2F1, could, however, result in the BLGmdm2 phenotype. MDM2 can bind Rb

and ultimately decrease its ability to arrest cells in G₁ in tissue culture (40). Although it would be of interest to test genetically the relevance of this interaction in generating the BLGmdm2 phenotype, the *Rb*^{-/-} mouse dies during embryogenesis (41, 42), and there are no available mice overproducing Rb in the appropriate tissue. MDM2 can also bind E2F1/DP1 and increase its ability to activate transcription of S-phase genes in tissue culture cells (11). We, therefore, tested the possibility of an MDM2 interaction with E2F1 as the likely factor in creating the BLGmdm2 phenotype. By crossing BLGmdm2 mice with mice either lacking or overexpressing *E2F1*, we were able to determine that there was no requirement for E2F1 in the BLGmdm2 phenotype.

MDM2 could regulate E2F1 function by a variety of mechanisms (see Fig. 1 for model). For example, it could lift the Rb/E2F1-mediated transcriptional repression of S-phase genes, it could encourage dissociation of Rb from E2F1, or it could act directly with E2F1 to activate S-phase genes. The ultimate result of all of these activities would be the promotion of S phase, one of the key findings in the mammary gland of mice overproducing MDM2. Therefore, we considered it quite possible that MDM2 was acting on E2F1 to cause inappropriate DNA synthesis. However, no changes were identified in BLGmdm2 mice in the presence or absence of *E2F1*, indicating that the promiscuous activation of endogenous E2F1 by MDM2 was not a critical factor in the development of the BLGmdm2 phenotype. Due to the multiplicity of E2F transcription factors, it is possible that endogenous E2F1 is not an essential regulator of the cell cycle in the epithelial cells of the mammary gland but that a different family member performs such a function.

Because E2F1 appears to have dual functions and too little or too much E2F1 can lead to cell cycle defects, we also tested whether overproduction of E2F1 in conjunction with MDM2 could exacerbate the phenotype of BLGmdm2 transgenic mice. We tested this possibility *in vivo* by generating mice that overproduce both MDM2 and E2F1. Again, we did not see an increase in the severity of the BLGmdm2 pheno-

type, nor did we see any major differences in the BLGE2F1 phenotype (see Fig. 5). Instead, we saw a unique mixture of the two phenotypes. This observation is interesting because it demonstrates that neither protein is more dominant than the other one. These data, in combination with the lack of alterations of the BLGmdm2 phenotype in either the absence or overexpression of E2F1 described in this report, lead to the conclusion that these two cell cycle regulators act independently of each other in this tissue.

Although we have focused our discussion on MDM2-interacting proteins, the possibility also exists that it is the ability of MDM2 to function as a transcription factor that results in the BLGmdm2 phenotype. MDM2 has many characteristics of transcription factors, including an acidic region, a nuclear localization signal, and a RING finger (43), and it can activate transcription when fused to the Lex A DNA-binding domain (44). Thus, the overexpression of *mdm2* in the mouse mammary gland may result in the alteration of gene expression.

MDM2, therefore, appears to be acting through a novel mechanism, when it is overproduced in the mammary gland of mice during pregnancy and lactation, to induce the phenotype of hypoproliferation, unregulated DNA synthesis, and polyploidy. Our findings effectively rule out interactions with both p53 and E2F1 as causes. What remains to be defined is the pathway by which MDM2 disrupts the normal proliferation and development of the mammary gland. Experiments addressing potential MDM2 transcriptional regulation or identifying novel MDM2-interacting proteins could possibly clarify this currently undefined pathway.

Materials and Methods

Mouse Breeding and Genotyping. BLGmdm2 transgenic mice (line TG3640; Ref. 9) were maintained as hemizygotes by crossing to C57BL/6J wild-type mice. 129/Sv-C57BL/6 mice heterozygous for a mutant E2F1 allele (*E2F1*^{+/-}) were obtained from L. Yamasaki (University of Columbia, New York, NY; Ref. 25) and crossed with mice carrying the BLGmdm2 transgene. BLGmdm2/*E2F1*^{+/-} progeny were back-crossed with *E2F1*^{+/-} mice to generate offspring that were BLGmdm2/*E2F1*^{+/-}. BLGE2F1 transgenic mice (line TG3604) were crossed with BLGmdm2 mice (line TG3640) to create mice that carried both transgenes in a hemizygous state.

The presence of the BLGmdm2 transgene was determined by a dominant coat color marker that had been coinjected with the transgene and by PCR with transgene-specific primers BLG and MDM2, as described previously (9). *E2F1* heterozygous and homozygous mutant animals were identified by PCR, as described previously (25), with the following exceptions. PCR was performed for both wild-type and mutant alleles together using 16 pmol each of L26 and L28 primers and 32 pmol of L31 primer per reaction. The PCRs (25 μ l) were amplified using AmpliTaq (Perkin-Elmer) for 1 cycle (94°C 5 min), 35 cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min), and 1 cycle (72°C for 7 min). BLGE2F1 transgenic mice were identified by D. M. B.⁵

Nulliparous females of the appropriate genotypes were mated and sacrificed at day 14 or 18 of gestation or at day 10 of lactation. If necessary, pups born to BLGmdm2 females were removed to a foster mother and replaced with slightly older pups to allow for continued nursing. Most animals were given an i.p. injection of 100 μ g of BrdUrd in PBS per g of body weight ~2 h before sacrifice. The first abdominal (number 4) mammary glands on both sides of the animal were dissected for analysis.

Immunohistochemistry. Mammary gland tissues were fixed in 0.4% paraformaldehyde in PBS at 4°C overnight, washed twice in PBS, and dehydrated through a graded series of ethanols, from 70 to 100%, ac-

cording to standard procedures. The tissue was then incubated in xylene for 30 min before it was embedded in paraffin. Sections were cut at 7 μ m and placed on lysine-coated slides. After rehydration, the slides were incubated in 0.01 M citrate buffer (pH 7.0) in a steamer for 25 min for antigen retrieval and then soaked in 0.3% hydrogen peroxide in methanol for 15 min. After rinsing in PBS, the slides were blocked with serum from the Vectastain kit (Vector Laboratories, Burlingame, CA) and then incubated with the appropriate antibody for 1 h at 37°C in a humidified chamber. MDM2 (9312) and cyclin E (1014) antibodies were rabbit polyclonal antisera raised by our laboratory and used at a 1:250 dilution. Cyclin A (C-19; polyclonal; 1:250) and cyclin D1 (72-13G; monoclonal; 1:100) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cyclin B (Santa Cruz Biotechnology and Upstate Biotechnology, Inc.), MPM-2 (Upstate Biotechnology, Inc.), and H3P (a gift from David Allis, University of Virginia Health Sciences Center, Charlottesville, VA) were also used at 1:100, 1:200, and 1:500 dilutions, respectively. All immunohistochemistry was performed with the Elite Vectastain Kit for mouse or rabbit (Vector Laboratories) according to the manufacturer's instructions. Staining was detected with the substrate DAB (Vector Laboratories). All immunostained slides were counterstained with Nuclear Fast Red (Vector Laboratories) before dehydrating and mounting with Permount. BrdUrd immunostaining was performed using the BrdUrd staining kit (Zymed, San Francisco, CA) according to the manufacturer's instructions.

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References

1. Momand, J., Zambetti, G. P., Olson D. C., George, D., and Levine, A. J. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53 mediated transactivation. *Cell*, 69: 1237-1245, 1992.
2. Barak, Y., Juven, T., Haffner, R., and Oren, M. *mdm2* expression is induced by wild type p53 activity. *EMBO J.*, 12: 461-468, 1993.
3. Wu, X., Bayle, J. H., Olson, D., and Levine, A. J. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.*, 7: 1126-1132, 1993.
4. Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature (Lond.)*, 378: 203-206, 1995.
5. Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. Rescue of early embryonic lethality in mdm2-deficient mice by absence of p53. *Nature (Lond.)*, 378: 206-208, 1995.
6. Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature (Lond.)*, 358: 80-83, 1992.
7. Cordon-Cardo, C., Latres, E., Drobnjak, M., Oliva, M. R., Pollack, D., Woodruff, J. M., Marechal, V., Chen, J., Brennan, M. F., and Levine, A. J. Molecular abnormalities of *mdm2* and *p53* genes in adult soft tissue sarcomas. *Cancer Res.*, 54: 794-799, 1994.
8. Dubs-Poterszman, M. C., Tocque, B., and Wasyluk, B. MDM2 trans-formation in the absence of p53 and abrogation of the p107 G₁ cell-cycle arrest. *Oncogene*, 11: 2445-2449, 1995.
9. Lundgren, K., Montes de Oca Luna, R., McNeill, Y. B., Emerick, E. P., Spencer, B., Barfield, C. R., Lozano, G., Rosenberg, M. P., and Finlay, C. A. Targeted expression of MDM2 uncouples S phase from mitosis and inhibits mammary gland development independent of p53. *Genes Dev.*, 11: 714-725, 1997.
10. Harris, S., McClenaghan, M., Simons, J. P., Ali, S., and Clark, A. J. Developmental regulation of the sheep β -lactoglobulin gene in the mammary gland of transgenic mice. *Dev. Genet.*, 12: 299-307, 1991.
11. Martin, K., Trouche, D., Hagemeier, C., Sorensen, T. S., LaThangue, N. B., and Kouzarides, T. Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature (Lond.)*, 375: 691-694, 1995.
12. Bagchi, S., Weinmann, R., and Raychaudhuri, P. The retinoblastoma protein copurifies with E2F-1, an E1A-regulated inhibitor of the transcription factor E2F. *Cell*, 65: 1063-1072, 1991.

13. Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. The E2F transcription factor is a cellular target for the RB protein. *Cell*, 65: 1053–1061, 1991.
14. Chittenden, T., Livingston, D. M., and Kaelin, W. G., Jr. The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. *Cell*, 65: 1073–1082, 1991.
15. Sherr, C. J. G₁ phase progression: cycling on cue. *Cell*, 79: 551–555, 1994.
16. Weinberg, R. A. The retinoblastoma protein and cell cycle control. *Cell*, 81: 323–330, 1995.
17. DeGregori, J., Kowalik, T., and Nevins, J. R. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G₁/S-regulatory genes. *Mol. Cell. Biol.*, 15: 4215–4224, 1995.
18. Blake, M. C., and Azizkhan, J. C. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene *in vitro* and *in vivo*. *Mol. Cell. Biol.*, 9: 4994–5002, 1989.
19. Mudryj, M., Hiebert, S. W., and Nevins, J. R. A role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal transduction pathway. *EMBO J.*, 9: 2179–2184, 1990.
20. Dou, Q. P., Markell, P. J., and Pardee, A. B. Thymidine kinase transcription is regulated at G₁/S phase by a complex that contains retinoblastoma-like protein and a cdc2 kinase. *Proc. Natl. Acad. Sci. USA*, 89: 3256–3260, 1992.
21. Pearson, B. E., Nasheuer, H. P., and Wang, T. S. Human DNA polymerase α gene: sequences controlling expression in cycling and serum-stimulated cells. *Mol. Cell. Biol.*, 11: 2081–2095, 1991.
22. Johnson, D. G., Schwartz, J. K., Cress, W. D., and Nevins, J. R. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature (Lond.)*, 365: 349–352, 1993.
23. Qin, X.-Q., Livingston, D. M., Kaelin, W. G., Jr., and Adams, P. D. Deregulated transcription factor E2F1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl. Acad. Sci. USA*, 91: 10918–10922, 1994.
24. Kowalik, T. F., DeGregori, J., Schwarz, J. D., and Nevins, J. R. E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. *J. Virol.*, 69: 2491–2500, 1995.
25. Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E., and Dyson, N. J. Tumor induction and tissue atrophy in mice lacking E2F1. *Cell*, 85: 537–548, 1996.
26. Field, S. J., Tsai, F.-Y., Kuo, F., Zubiaga, A. M., Kaelin, W. G., Jr., Livingston, D. M., Orkin, S. H., and Greenberg, M. E. E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell*, 85: 549–561, 1996.
27. Wu, X., and Levine, A. J. p53 and E2F-1 cooperate to mediate apoptosis. *Proc. Natl. Acad. Sci. USA*, 91: 3602–3606, 1994.
28. Weintraub, S. J., Prater, C. A., and Dean, D. C. Retinoblastoma protein switches the E2F site from positive to negative element. *Nature (Lond.)*, 358: 259–261, 1992.
29. Weintraub, S. J., Chow, K. N. B., Luo, R. X., Zhang, S. H., He, S., and Dean, D. C. Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature (Lond.)*, 375: 812–815, 1995.
30. Adnane, J., Shao, Z., and Robbins, P. D. The retinoblastoma susceptibility gene product represses transcription when directly bound to the promoter. *J. Biol. Chem.*, 270: 8837–8843, 1995.
31. Sellers, W. R., Rodgers, J. W., and Kaelin, W. G., Jr. A potent *trans*-repression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. *Proc. Natl. Acad. Sci. USA*, 92: 11544–11548, 1995.
32. Davis, F. M., Tsao, T. Y., Fowler, S. K., and Rao, P. N. Monoclonal antibodies to mitotic cells. *Proc. Natl. Acad. Sci. USA*, 80: 2926–2930, 1983.
33. Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P., and Allis, C. D. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G₂ and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma*, 106: 348–360, 1997.
34. Bortner, D. M., and Rosenberg, M. P. Overexpression of cyclin A in the mammary glands of transgenic mice results in the induction of nuclear abnormalities and increased apoptosis. *Cell Growth Differ.*, 6: 1579–1589, 1995.
35. Haupt, Y., Barak, Y., and Oren, M. Cell type-specific inhibition of p53-mediated apoptosis by mdm2. *EMBO J.*, 15: 1596–1606, 1996.
36. Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosfeld, G., and Sherr, C. J. Tumor suppression at the mouse Ink4a locus mediated by the alternative reading frame product p19ARF. *Cell*, 91: 649–659, 1997.
37. Pomerantz, J., Schreiber-Agus, N., Liegeois N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlov, I., Lee, H.-W., Cordon-Cardo, C., and DePinho, R. A. The Ink4a tumor suppressor gene product, p19ARF, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell*, 92: 713–723, 1998.
38. Zhang, Y., Xiong, Y., and Yarbrough, W. G. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell*, 92: 725–734, 1998.
39. Juven-Gershon, T., Shifman, O., Unger, T., Elkeles, A., Haupt, Y., and Oren, M. The Mdm2 oncoprotein interacts with the cell fate regulator Numb. *Mol. Cell. Biol.*, 18: 3974–3982, 1998.
40. Xiao, Z.-X., Chen, J., Levine, A. J., Modjtahedi, N., Xing, J., Sellers, W. R., and Livingston, D. M. Interaction between the retinoblastoma protein and the oncoprotein MDM2. *Nature (Lond.)*, 375: 694–698, 1995.
41. Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., and Weinberg, R. A. Effects of an Rb mutation in the mouse. *Nature (Lond.)*, 359: 295–300, 1992.
42. Lee, E. Y., Chang, C.-Y., Hu, N., Wang, Y.-C. J., Lai, C.-C., Herrup, K., Lee, W.-H., and Bradley, A. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature (Lond.)*, 359: 288–294, 1992.
43. Lozano, G., and Montes de Oca Luna, R. MDM2 function. *Biochim. Biophys. Acta*, 1377: M55–M59, 1998.
44. Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyruis, J., Kinzler, K. W., and Vogelstein, B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature (Lond.)*, 362: 857–860, 1993.

LOSS OF ONE BUT NOT TWO *mdm2* NULL ALLELES ALTERS THE TUMOUR SPECTRUM IN *p53* NULL MICE

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SUMMARY

The transcriptional activity of the *p53* tumour suppressor is inhibited by binding to MDM2. The *in vivo* significance of this interaction was established in *mdm2* null mice. Embryonic lethality due to loss of *mdm2* is completely rescued by deletion of *p53*, indicating that the lethality is due to inability to down-modulate *p53* function. The production of mice null for both *p53* and *mdm2* led to an assessment of the role of MDM2 in tumour development. Tumour latency and spectrum in *p53* null mice were monitored in the presence or absence of *mdm2*. Two unusual findings resulted: tumour latency in *p53* null/*mdm2* heterozygous mice was longer than in *p53/mdm2* double-null mice; and the incidence of sarcomas was higher in *p53* null/*mdm2* heterozygous mice than in *p53* null or *p53/mdm2* double-null mice. These data raise the possibility that heterozygosity at the *mdm2* locus in the absence of *p53* affects the development of tumours of mesenchymal origin. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS—sarcomas; lymphomas; tumour suppressor genes; transgenic mice; *p53*; *mdm2*; mouse models

INTRODUCTION

Mutation of the tumour suppressor *p53* negates its ability to arrest the cell cycle and to induce apoptosis, and is the most common alteration observed in tumours.^{1–4} *p53* function can also be altered by amplification of the *mdm2* gene, which has been observed in approximately one-third of sarcomas and to a lesser extent in glioblastomas and breast carcinomas.^{5–7} Since MDM2 binds *p53* and inhibits *p53* function,⁸ these data have led to the hypothesis that amplification of *mdm2* is an alternative mechanism of inactivating *p53*.

MDM2 binds the amino-terminal transactivation domain of *p53*, inhibiting its interaction with the transcriptional apparatus.^{9–11} Mutation of *p53* amino acids leu22 and trp23 or MDM2 amino acids gly58, glu68, val75, or cys77 results in lack of binding, limiting the interaction motif to the amino terminus of both proteins.¹² The crystal structure of the *p53*/MDM2 complex indicated an unusual interaction motif that is primarily dependent on van der Waals forces, with only two hydrogen bonds.¹³ The binding of MDM2 to *p53* also promotes the rapid degradation of *p53*.^{14,15}

The importance of the inhibition of *p53* transcriptional activity by MDM2 is most clearly exemplified in the analysis of *mdm2* function *in vivo*.^{16,17} The deletion of *mdm2* in mice leads to early embryonic lethality, at

5.5 days of development. This phenotype is completely rescued in the absence of *p53*, suggesting that lethality is due to the inability to down-modulate *p53* activity during embryonic development.

These data, however, do not preclude alternative functions for MDM2 and several observations suggest that it may have functions independent of *p53*. For example, some tumours contain both a mutation of the *p53* gene and overproduction of MDM2, suggesting that in cells, these two genetic changes do not have identical effects.¹⁸ In addition, overexpression of *mdm2* leads to cell-cycle defects in breast epithelial cells.¹⁹ The enlarged and multinucleated *mdm2*-expressing epithelial cells undergo multiple rounds of DNA synthesis without cytokinesis. More important, the absence of *p53* has no effect on this phenotype. The *p53*-independent functions of MDM2 may be attributed to the fact that MDM2 binds other proteins such as Rb and E2F1.^{20,21} The binding of MDM2 to Rb prevents its function as an inhibitor of the cell cycle and the binding to E2F1 augments E2F1 transcriptional activity during S-phase progression. In addition, MDM2 binds L5, a member of the ribosome complex, and its associated 5S ribosomal RNA.^{22,23} Together, these observations indicate additional functions for MDM2 other than solely inactivation of *p53*.

These findings led us to analyse mice null for *p53* and *mdm2* for tumourigenesis. Tumour latency was prolonged in *p53* null/*mdm2* heterozygous mice, compared with *p53/mdm2* double-null mice. In addition, the tumour spectrum also changed, with an increased number of sarcomas in *p53* null/*mdm2* heterozygous mice, compared with *p53* null and *p53/mdm2* double-null mice. To our knowledge, this is the first example of a phenotype that results from having only one functional allele which disappears upon deletion of the other

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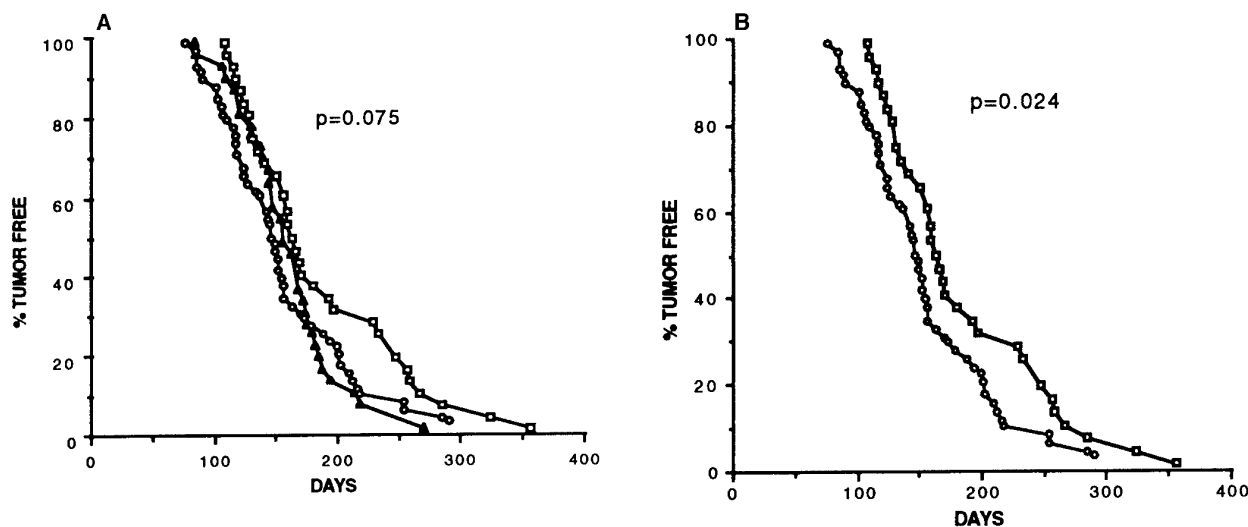


Fig. 1—Effects of *mdm2* deletion on tumour latency in *p53* mice. Thirty-four *mdm2*^{+/+} (solid triangles), 33 *mdm2*^{+/-} (squares), and 58 *mdm2*^{-/-} mice (circles), all null for *p53* and with tumours, were used to calculate tumour latency. The percentage of mice remaining tumour-free is plotted against age. The *p* value determined using Kaplan-Meier analysis is shown for all three genotypes in A and for *p53*^{-/-} *mdm2*^{+/-} (squares) and *p53*^{-/-} *mdm2*^{-/-} (circles) mice in B

normal allele. This may be attributed to the unique ability of MDM2 to inhibit p53 function and to interact with other proteins. These data further support an additional role for MDM2 other than inhibition of p53 function.

MATERIALS AND METHODS

Mice

Mice with three different genotypes generated by gene targeting were used in this study. All of the mice were null for *p53*²⁴ and were either wild-type, heterozygous, or homozygous for the *mdm2* null allele.¹⁶ To generate these genotypes, mice heterozygous for the *mdm2* null mutation were crossed with normal C57BL/6J females to produce two generations of mice of mixed 129/Sv:C57BL/6 background. These mice were crossed with 129/Sv *p53* null mice²⁴ (obtained from Jackson Laboratory) to generate mice heterozygous for both mutant alleles. These double-heterozygote mice were crossed with each other to obtain the mice used in this tumorigenic study. Genotyping for each of the null alleles was performed by the polymerase chain reaction (PCR) as described previously.^{16,24}

Tumour analysis

Mice that developed visible tumours approximately 1 cm in diameter were killed and subjected to necropsy. Mice that did not develop visible tumours but became moribund were also killed and subjected to necropsy. In addition to tumour samples, tissues from heart, lung, kidney, spleen, liver, and testis were recovered. The mice were carefully examined for the presence of any other abnormalities. Brain samples were taken in those few cases from moribund mice with no apparent pathology

or tumour. All the tissues were fixed in 10 per cent buffered formalin, processed for histology, and paraffin-embedded. Four-micrometre-thick paraffin sections were stained with haematoxylin and eosin. The histopathological analysis was performed without knowledge of the genotypes of the mice.

Statistical analysis

Comparison of the tumour latency for the three strains of mice was performed using Kaplan-Meier analysis. Significant differences in the type and dissemination of tumours between mice of the three genotypes were established using the chi-square test.

RESULTS

p53 null mice are prone to the development of multiple types of tumours early in their lives.^{24,25} Our ability to rescue mice null for *mdm2* in a *p53* null background prompted us to examine the contribution of *mdm2* to tumorigenesis in *p53* null mice. We wished to assess the influence of the absence of one or two *mdm2* alleles on the latency and pathogenesis of neoplastic disease in *p53* null mice. Crosses were performed using mice with different combinations of *p53* and *mdm2* null alleles as described in the Materials and Methods to generate the following cohort: *p53* null mice (*n*=34); *p53* null/*mdm2* heterozygous mice (*n*=33); and *p53*/*mdm2* double-null mice (*n*=58). The mice used in this study were in a mixed C57/129 background.

To measure tumour incidence, we plotted the number of animals that remained tumour-free against time (Fig. 1A). The mean latency for *p53*^{-/-} *mdm2*^{+/+}, *p53*^{-/-} *mdm2*^{+/-}, and *p53*^{-/-} *mdm2*^{-/-} mice was 150.5, 156.5, and 140.5 days, respectively. Latency

Table I—Tumour spectrum in *mdm2* null, heterozygous, and wild-type mice in a *p53* null background

	<i>mdm2</i> +/+	<i>mdm2</i> +/-	<i>mdm2</i> -/-
Lymphoma	29* (85%)†	21 (64%)	52 (90%)
Sarcoma			
Spindle cell sarcoma	6 (18%)	6 (18%)	5 (9%)
Angiosarcoma	2 (6%)	7 (21%)	4 (7%)
Osteosarcoma	—	2 (6%)	1 (2%)
Multiple neoplasms	5 (15%)	8 (24%)	8 (14%)
Teratoma	1 (3%)	1 (3%)	2 (3%)
Carcinoma	1 (3%)	2 (6%)	—
Teratocarcinoma	—	—	2 (3%)
Other neoplasms	1 (3%)	1 (3%)	1 (2%)

Thirty-four *mdm2*+/+, 33 *mdm2*+/-, and 58 *mdm2*-/- mice all of them null for *p53*, that had developed a tumour were subjected to histopathological analysis to identify the type of tumour.

*One mouse had two lymphomas.

†The percentages for each genotype will not equal 100 per cent, since multiple tumours arose in some mice.

in the *p53*-/- *mdm2*+/- mice was significantly longer than in *p53*-/- *mdm2*-/- mice ($p=0.024$) (Fig. 1B).

Lymphomas

We also performed a pathological examination of the tumours that arose in the three genotypic strains of mice. Most tumours arising in the mice of each genotype were lymphomas, constituting approximately 85 per cent of the tumours in the *p53*-/- mice and 90 per cent in the *p53*-/- *mdm2*-/- mice (Table I). Although lymphomas were the most common malignancy in the *p53*-/- *mdm2*+/- mice (64 per cent), they were significantly less common than in mice of the other two genotypes ($p=0.007$) (Fig. 2). The majority of lymphomas were thymic in origin; this type constituted 73 per cent of the lymphomas in the *p53*-/- mice, 83 per cent of the lymphomas in the *p53*-/- *mdm2*-/- mice, and 90 per cent of the lymphomas in the *p53*-/- *mdm2*+/- mice (Table II). There was no significant difference in latency to detection of lymphomas arising in the three genetic backgrounds. The thymic lymphomas in all three genotypes were high-grade large-cell malignancies with numerous mitotic figures and evidence of apoptotic cell death (Fig. 3A).

The thymic lymphomas exhibited a characteristic pattern of dissemination and organ involvement that in general was similar in each genotypic background. In every case examined, there was histological evidence of dissemination, most commonly to sites above the diaphragm (Table II). Most also involved the heart (Table II); the incidence of cardiac involvement was significantly lower in the *p53*-/- *mdm2*+/- mice than in mice of the other genotypes ($p=0.039$) (Fig. 4). Characteristically, cardiac involvement was limited to an expansion of the epicardial space (Fig. 3B) and appreciable infiltration of the myocardium or endocardial involvement was not observed. The significance of the

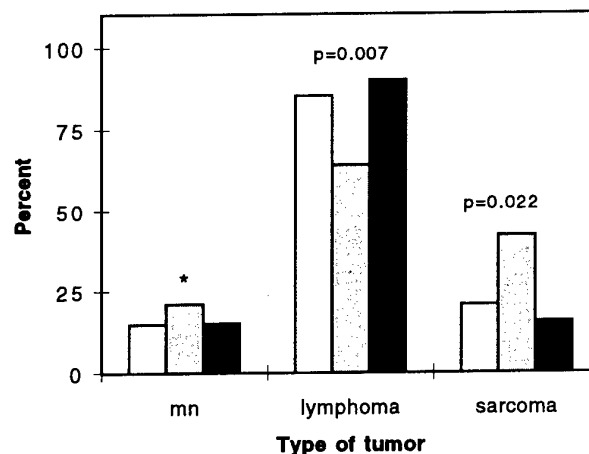


Fig. 2—Effects of *mdm2* mutation on tumour development in *p53* null mice. The frequency of lymphomas, sarcomas, and mice with multiple neoplasms (mn) were compared between the different genetic backgrounds. The frequency of each type of tumour is relative to the total numbers of tumours observed in each genotype. Comparison of the frequencies between tumour type and genetic background by chi-square analysis indicates a statistically significant difference in the incidence of lymphomas and sarcomas, $p=0.007$ and $p=0.022$, respectively, in *p53*-/- *mdm2*+/- mice compared with the other two genotypes. White, grey, and black bars represent *p53*-/- *mdm2*+/+, *p53*-/- *mdm2*+/-, and *p53*-/- mice, respectively.

lower incidence of cardiac involvement in thymic lymphomas arising in *p53*-/- *mdm2*+/- mice is unclear, although it may indicate that these tumours are somewhat less aggressive locally than their counterparts arising in other genetic backgrounds.

The lung was the most common site of extrathymic lymphoma involvement, representing 84 per cent of the cases in the *p53*-/- mice, 80 per cent in *p53*-/- *mdm2*+/- mice, and 95 per cent in *p53*-/- *mdm2*-/- mice (Table II). Pulmonary involvement characteristically resulted in multifocal expansion of the bronchovascular bundles by lymphomas (Fig. 3C). More extensive involvement was characterized by the progressive expansion of the alveolar septa and eventual filling of the alveolar spaces.

The most common site of extrathymic lymphomas involvement below the diaphragm was the liver (Fig. 3D). Hepatic involvement was observed in 50 per cent of the cases in *p53*-/- mice, 53 per cent in *p53*-/- *mdm2*+/- mice, and 69 per cent in *p53*-/- *mdm2*-/- mice (Table II). Typically, multifocal expansion of the portal tracts was observed. Expansion of the hepatic sinusoids by lymphoma was characteristic of more extensive involvement. Approximately one-third of the cases of thymic lymphomas exhibited renal involvement (Table II). In most cases, renal involvement centred around the interlobular vessels. Occasionally, lymphomatous expansion of the renal hilum was observed, centred around the hilar vasculature. Approximately 20–30 per cent of the thymic lymphomas exhibited splenic involvement (Table II). Characteristically, this resulted in splenomegaly due to extensive lymphomatous infiltration of the red pulp (Fig. 3E). The splenic architecture was occasionally disrupted.

Table II—Incidence of lymphoma and its dissemination

	Heart	Lung	Liver	Spleen	LN	Kidney
<i>p53</i> -/- <i>mdm2</i> +/+						
Thymic (22 cases)	18/20 (90%)*	16/19 (84%)	10/20 (50%)	4/20 (20%)	2/2 (100%)	6/20 (30%)
Splenic (7 cases)	0/5	3/6 (50%)	5/5 (100%)	7/7 (100%)	1/1 (100%)	3/6 (50%)
Peripheral (1 case)	—	—	+	—	+	—
<i>p53</i> -/- <i>mdm2</i> +/-						
Thymic (19 cases)	10/18 (56%)	12/15 (80%)	9/17 (53%)	4/18 (22%)	0/1	5/18 (28%)
Splenic (1 case)	—	+	+	+	+ (MLN)	+
Peripheral (1 case)	+	+	+	+	+	+
<i>p53</i> -/- <i>mdm2</i> -/-						
Thymic (44 cases)	33/42 (79%)	35/37 (95%)	24/35 (69%)	10/31 (32%)	1/1 (100%)	12/37 (32%)
Splenic (6 cases)	0/6	2/5 (40%)	5/5 (100%)	6/6 (100%)	4/5 (80%)	4/5 (80%)
Peripheral (3 cases)	2/3 (67%)	2/3 (67%)	2/3 (67%)	3/3 (100%)	3/3 (100%)	3/3 (100%)

*Only a fraction of the total number of samples were analysed for dissemination.

LN=lymph node.

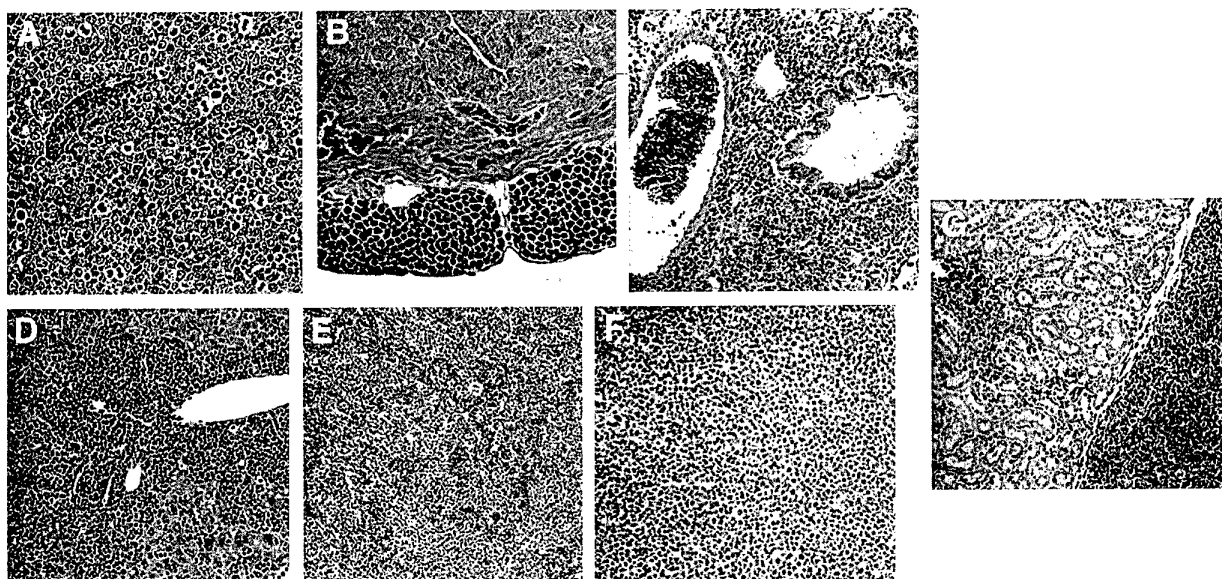


Fig. 3—Histopathological features of malignant lymphomas arising in *p53* -/- *mdm2* -/- and *p53* -/- *mdm2* +/- mice. (A) Thymic lymphoma arising in a *p53* -/- *mdm2* -/- mouse showing the characteristically high rates of apoptosis and mitosis. (B) Section of heart showing epicardial involvement by thymic lymphoma, with minimal involvement of the myocardium. (C) Pulmonary involvement by thymic lymphoma, showing expansion of the bronchovascular bundle with proximal extension into the alveolar septa. (D) Hepatic involvement by lymphoma, showing expansion of the portal tract and sinusoidal infiltration. (E) Disseminated thymic lymphoma, involving preferential infiltration of the splenic red pulp. (F) Primary splenic lymphoma, showing replacement of splenic white pulp by high-grade large cell lymphoma. (G) Renal dissemination of splenic lymphoma, showing the characteristic capsular involvement. A focus of lymphoma showing expansion of interlobular vascular spaces is also present in the renal cortex.

Splenic lymphomas constituted 23 per cent of the lymphomas arising in the *p53* -/- mice, 5 per cent of the lymphomas in the *p53* -/- *mdm2* +/- mice, and 11 per cent of lymphomas in the *p53* -/- *mdm2* -/- mice (Table II). In contrast to secondary involvement of the spleen by thymic lymphomas, these malignancies were centred in the white pulp (Fig. 3F). The histopathological features were consistent with high-grade large-cell lymphoma and in all cases, there was histologically confirmed evidence of dissemination. Dissemination most commonly involved sites below the diaphragm, most often the liver and kidney. Splenic lymphomas involved the lung 50 per cent of the time in *p53* -/-

mice, 40 per cent in *p53* -/- *mdm2* -/- mice, and 100 per cent (one case) in *p53* -/- *mdm2* +/- mice. Dissemination of splenic lymphoma to the heart was not observed. The histological pattern of organ infiltration was similar to that of thymic lymphomas, except that capsular infiltration by retroperitoneal disease was a common feature of renal involvement by splenic lymphoma (Fig. 3G).

The remainder of the lymphomas were nodal lymphomas which exhibited the histological features of high-grade large-cell malignancies (Table II). These malignancies invariably demonstrated extranodal extension.

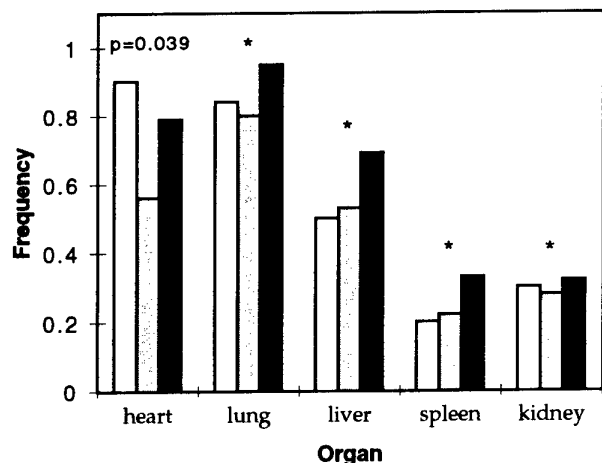


Fig. 4—Effects of *mdm2* mutation on lymphoma dissemination in *p53* null mice. Twenty-two *mdm2*^{+/+}, 19 *mdm2*^{+/-}, and 44 *mdm2*^{-/-} mice, all of them null for *p53*, that had developed lymphoma were used to determine the tumour dissemination. Heart, lung, liver, spleen, kidney, and testis were fixed and examined by histopathology as described. Comparison of the frequencies between organ involvement and genetic background was performed using chi square analysis and is depicted above the appropriate bars. *Not significant. White, grey, and black bars represent *p53*^{-/-} *mdm2*^{+/+}, *p53*^{-/-} *mdm2*^{+/-}, and *p53*^{-/-} *mdm2*^{-/-} mice, respectively

Sarcomas

Sarcomas constituted the next most common malignancy, accounting for approximately 24 per cent of the neoplasms arising in *p53*^{-/-} mice and 17 per cent of the neoplasms arising in *p53*^{-/-} *mdm2*^{-/-} mice (Table I). Sarcomas were significantly more common in the *p53*^{-/-} *mdm2*^{+/-} mice, comprising 45 per cent of the neoplasms ($p=0.022$) (Fig. 2). The mean latency period for sarcomas ranged from 183 to 195 days and was not significantly different in the three genotypic strains of mice.

Fibrosarcomas represented the most common histological variant (Fig. 5A). These tumours were characterized by spindle cells frequently arranged in a fasciculated pattern. Mitotic figures were frequent. Cellular pleomorphism was minimal, but occasional tumours exhibited foci of tumour giant cells (Fig. 5B). These tumours most commonly arose in subcutaneous connective tissue and attained diameters of 1–3 cm. They were locally aggressive and invaded underlying skeletal muscle, but metastases were not observed.

Angiosarcomas were the next most common sarcoma. Interestingly, 12 out of the total of 13 cases of angiosarcoma developed in female mice. These tumours were relatively small (0.2–0.5 cm) and grossly haemorrhagic. Most were intra-abdominal and fixed to the peritoneal membrane. Like fibrosarcomas, these tumours were locally invasive but not metastatic. Histologically, they were characterized by irregular anastomatic vascular channels (Fig. 5C). The endothelial lining of these vascular channels was markedly pleomorphic and numerous mitotic figures were observed.

Three cases of osteosarcoma were observed (Table I). Two developed in *p53*^{-/-} *mdm2*^{+/-} mice and one in

a *p53*^{-/-} *mdm2*^{-/-} mouse. Two were grossly consistent with extraskelatal osteosarcomas and one arose from a rib. These tumours were characterized by considerable cellular pleomorphism and haphazardly arrayed foci of osteoid and bone (Fig. 5D). All three osteosarcomas displayed locally invasive growth. Two of the three tumours were associated with metastatic lesions involving the liver and spleen.

Teratoma and teratocarcinoma

Four cases of teratoma and two cases of teratocarcinoma were diagnosed (Table I). Both teratocarcinomas were testicular in origin and occurred in *p53*^{-/-} *mdm2*^{-/-} mice. Three of the four teratomas were testicular and one was ovarian in origin. Three of the four teratomas arose in *p53*^{-/-} *mdm2*^{-/-} mice and one in each of the other two genotypes. The teratomas possessed solid and cystic elements and exhibited structures derived from ectoderm, mesoderm, and endoderm (Fig. 5E). Histologically, these structures included keratinizing epithelium, glandular epithelium, respiratory tissue, neuronal tissue, and bone. In both teratocarcinomas, the malignant component consisted of undifferentiated cells.

The remainder of the neoplasms consisted of unclassified malignant neoplasms (two cases) and myelogenous leukaemia (one case). A total of three carcinomas were observed: an adenocarcinoma of the submandibular gland, an adenocarcinoma of the pancreas, and an endometrial carcinoma.

DISCUSSION

In the experiments described in this report, we compared the tumour incidence in mice lacking *p53* that were also either wild-type, heterozygous, or null for *mdm2*. These experiments were aimed at determining the influence of *mdm2* on survival and the tumour spectrum of *p53* null mice. Several important observations were made: the latency to tumour development was longer for the *p53*^{-/-} *mdm2*^{+/-} mice than for the *p53*^{-/-} *mdm2*^{-/-} mice; significantly fewer lymphomas arose in *p53*^{-/-} *mdm2*^{+/-} mice than in *p53*^{-/-} or *p53*^{-/-} *mdm2*^{-/-} mice; significantly fewer of the thymic lymphomas involved the heart in *p53*^{-/-} *mdm2*^{+/-} mice than in *p53*^{-/-} mice; and sarcomas were significantly more common in *p53*^{-/-} *mdm2*^{+/-} mice. The possibility that this difference is due to genetic background is unlikely, due to the way in which we backcrossed these mice. Thus, mice null for *p53* and heterozygous for *mdm2* differed significantly from double-null mice, suggesting that heterozygosity at the *mdm2* locus altered not only the timing of tumour development, but also the tumour type.

The fact that the *p53*^{-/-} *mdm2*^{+/-} mice survive longer than *p53*^{-/-} *mdm2*^{-/-} mice suggests that MDM2 is a critical determinant of tumour suppression in the absence of *p53*. This clear gene dosage effect may best be addressed in the light of a new *in vitro* observation indicating that the full-length human or mouse

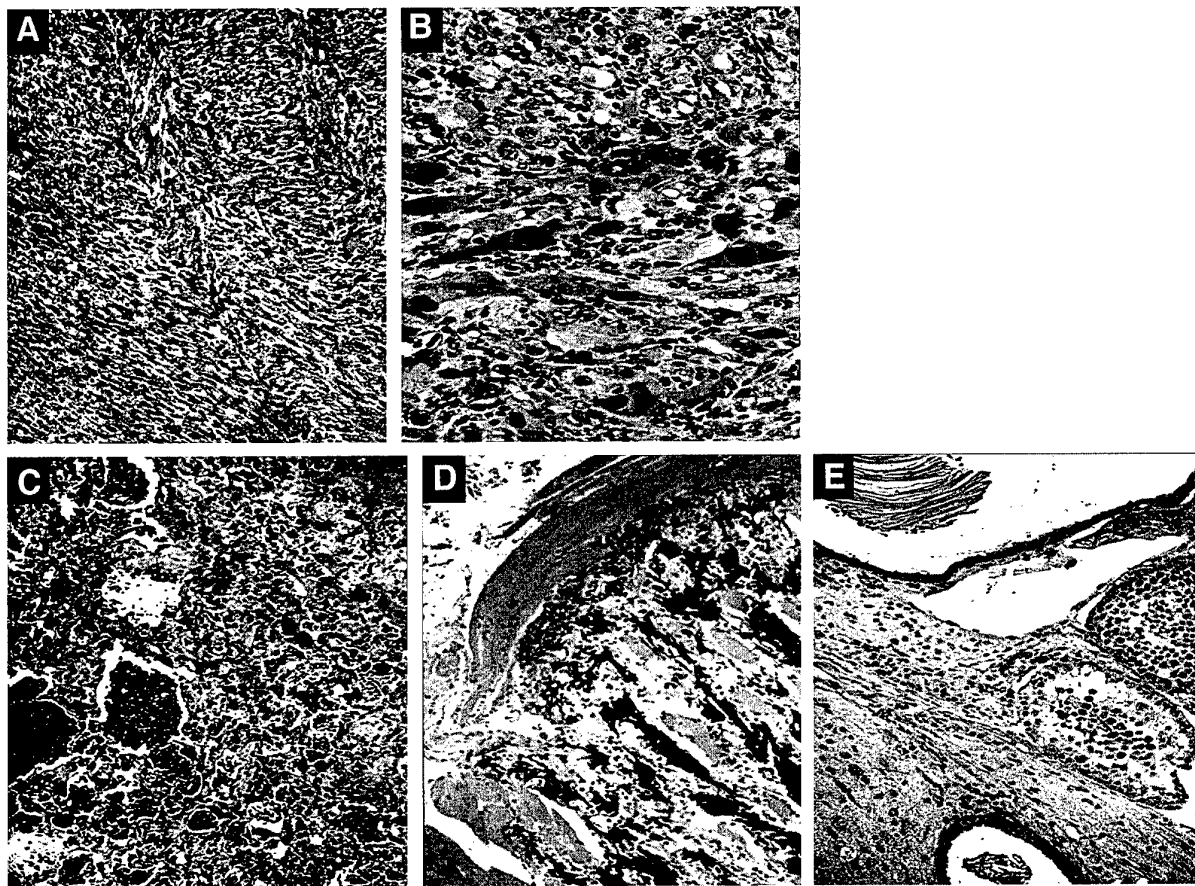


Fig. 5—Histopathological features of sarcomas. (A) Characteristic histological appearance of soft tissue fibrosarcoma, showing interlacing fascicles of spindle cells. (B) Occasional foci of tumour giant cells are observed in soft tissue sarcomas. (C) Angiosarcoma demonstrating an anastomosing pattern of vascular channels and considerable cellular pleomorphism. (D) Osteosarcoma arising in a $p53^{-/-}$ $mdm2^{+/-}$ mouse, showing nascent bone formation. (E) Testicular teratoma arising in a $p53^{-/-}$ $mdm2^{-/-}$ mouse, showing ectodermal and endodermal derived structures

MDM2 protein can inhibit the G0/G1–S phase transition of NIH3T3 and normal human diploid cells.²⁶ Therefore, inactivation of the MDM2-induced G0/G1 arrest may contribute to tumour development.

This function of *mdm2* is in contrast to previous *in vitro* and *in vivo* studies that clearly identified a role for *mdm2* in transformation and tumourigenesis.^{19,27} Increased levels of MDM2 due to gene amplification have been observed in sarcomas and other tumours.^{5–7} Moreover, the overexpression of *mdm2* in the breast epithelium of transgenic mice led to tumour development.¹⁹ Thus, *mdm2* appears to play dual roles, as a tumour suppressor and as an oncogene, depending on the levels of MDM2 being expressed in the cell. This role is reminiscent of the transcription factor E2F1, another MDM2-binding protein.²⁰ Multiple experiments indicate that E2F1 overexpression results in tumourigenesis.²⁸ However, loss of *E2F1* in mice also results in tumourigenesis.^{29,30} Thus, the balance of these positive and negative regulators of the cell cycle is critical for control of cell proliferation and small differences in the levels of any of these proteins may mean large differences in activities. Consistent with this hypothesis, tumour incidence in mice null for both *p53* and *mdm2*

was not significantly different from that of *p53* null mice. Thus, the complete absence of interactions of MDM2 with other proteins does not upset the balance of proteins involved in controlling cell proliferation.

Another important observation is the increased number of sarcomas in *p53* null/*mdm2* heterozygous mice, compared to *p53* null and *p53/mdm2* double-null mice. This observation further substantiates the critical nature of MDM2 levels in mesenchymal tissue. The amplification of the *mdm2* gene is seen more often in sarcomas than in any other tumour type.^{5–7} The increased incidence of spontaneous sarcomas in the $p53^{-/-}$ $mdm2^{+/-}$ mice suggests that an insufficient level, as well as an excess, of MDM2 could contribute to multistep carcinogenesis in a cell type-specific manner. An independent study using a different deletion of *mdm2* yielded no significant difference in sarcoma incidence between mice of these three genotypes.³¹ A tendency towards an increased incidence of sarcomas in $p53^{-/-}$ $mdm2^{+/-}$ mice compared with $p53^{-/-}$ mice was observed in that study, but was not statistically significant. The small number of mice used in that study could account for the lack of statistical significance.

This is an example of a phenotype present in heterozygous mice that is absent in null or normal mice and is probably due to the complex interactions between MDM2 and several proteins that are critical for the regulation of cell proliferation. Competition between these proteins and limiting amounts of MDM2 may result in the observed phenotype. The generation of mice with a targeted deletion of *mdm2* that would bypass the problem of embryo lethality should yield insight into this problem.

The clear *mdm2* gene dosage effect seen on the survival and tumour spectrum of *p53* null mice suggests that the transformation of normal cells to tumour cells is indeed a complicated process. Tumour cells arise by the stepwise accumulation of genetic alterations that ultimately result in loss of growth control. However, in this process some of the genetic alterations may actually take the cell a step back, towards a more normal phenotype. In our example, loss of *p53* and other unknown changes would result in a transformed cell whose phenotype might be partially reversed by the subsequent loss of one *mdm2* allele. It is interesting to note that some *p53* heterozygous mice develop tumours without loss of the remaining wild-type *p53* allele, again indicating that gene dosage may play a role in the rate at which tumours develop.³²

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REFERENCES

- Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997; **88**: 323–331.
- Ko LJ, Prives C. p53: puzzle and paradigm. *Genes Dev* 1996; **10**: 1054–1072.
- Greenblatt MS, Bennett WP, Holstein M, Haris CC. Mutations in the p53 tumour suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994; **54**: 4855–4878.
- Hainaut P, Soussi T, Shomer B, et al. Database of p53 gene somatic mutations in human tumours and cell lines: updated compilation and future prospects. *Nucleic Acids Res* 1997; **25**: 151–157.
- Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature* 1992; **358**: 80–83.
- Ladanyi M, Cha C, Lewis R, Jhanwar SC, Huvos AG, Healy JH. MDM2 gene amplification in metastatic osteosarcoma. *Cancer Res* 1993; **53**: 16–18.
- Momand J, Zambetti GP. Mdm-2: 'Big brother' of p53. *J Cell Biochem* 1997; **64**: 343–352.
- Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992; **69**: 1237–1245.
- Chen J, Marechal V, Levine AJ. Mapping of the p53 and mdm-2 interaction domains. *Mol Cell Biol* 1993; **13**: 4107–4114.
- Oliner JD, Pieterpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature* 1993; **362**: 857–860.
- Thut CJ, Goodrich JA, Tjian R. Repression of p53-mediated transcription by MDM2: a dual mechanism. *Genes Dev* 1997; **11**: 1974–1986.
- Freedman DA, Epstein CB, Roth JC, Levine AJ. A genetic approach to mapping the p53 binding site in the MDM2 protein. *Mol Med* 1997; **3**: 248–259.
- Kussie PH, Gorina S, Marechal V, et al. Structure of the MDM2 oncoprotein bound to the p53 tumour suppressor transactivation domain. *Science* 1996; **274**: 948–953.
- Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997; **387**: 296–299.
- Kubbutat MHG, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. *Nature* 1997; **387**: 299–303.
- Montes de Oca Luna R, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* 1995; **378**: 203–206.
- Jones SN, Roe AE, Donehower LA, Bradley A. Rescue of embryonic lethality in mdm2-deficient mice by absence of p53. *Nature* 1995; **378**: 206–208.
- Cordon-Cardo C, Latres E, Drobnjak M, et al. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. *Cancer Res* 1994; **54**: 794–799.
- Lundgren K, Montes de Oca Luna R, McNeill YB, et al. Targeted expression of MDM2 uncouples S phase from mitosis and inhibits mammary gland development independent of p53. *Genes Dev* 1997; **11**: 714–725.
- Xiao ZX, Chen J, Levine AJ, et al. Interaction between the retinoblastoma protein and the oncoprotein MDM2. *Nature* 1995; **375**: 694–698.
- Martin K, Trouche D, Hagemeier C, Sorensen TS, La Thangue NB, Kouzarides T. Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature* 1995; **375**: 691–694.
- Marechal V, Elenbaas B, Piette J, Nicolas JC, Levine AJ. The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes. *Mol Cell Biol* 1994; **14**: 7414–7420.
- Elenbaas B, Dobbelsstein M, Roth J, Shenk T, Levine AJ. The MDM2 oncoprotein binds specifically to RNA through its RING finger domain. *Mol Med* 1996; **2**: 439–451.
- Jacks T, Remington L, Williams BO, et al. Tumour spectrum analysis in p53-mutant mice. *Curr Biol* 1994; **4**: 1–7.
- Donehower LA, Harvey M, Slagle BL, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 1992; **356**: 215–221.
- Brown DR, Thomas CA, Deb SP. The human oncoprotein MDM2 arrests the cell cycle: elimination of its cell-cycle-inhibitory function induces tumorigenesis. *EMBO J* 1998; **17**: 2513–2525.
- Finlay CA. The mdm-2 oncogene can overcome wild-type p53 suppression of transformed cell growth. *Mol Cell Biol* 1993; **13**: 301–306.
- Weinberg RA. E2F and cell proliferation: a world turned upside down. *Cell* 1996; **85**: 457–459.
- Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E, Dyson NJ. Tumour induction and tissue atrophy in mice lacking E2F-1. *Cell* 1996; **85**: 537–548.
- Field SJ, Tsai F-Y, Kuo F, et al. E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* 1996; **85**: 549–561.
- Jones SN, Sands AT, Hancock AR, et al. The tumorigenic potential and cell growth characteristics of p53-deficient cells are equivalent in the presence or absence of Mdm2. *Proc Natl Acad Sci USA* 1996; **93**: 14 106–14 111.
- Venkatachalam S, Shi YP, Jones SN, et al. Retention of wild-type p53 in tumours from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *EMBO J* 1998; **17**: 4657–4667.